

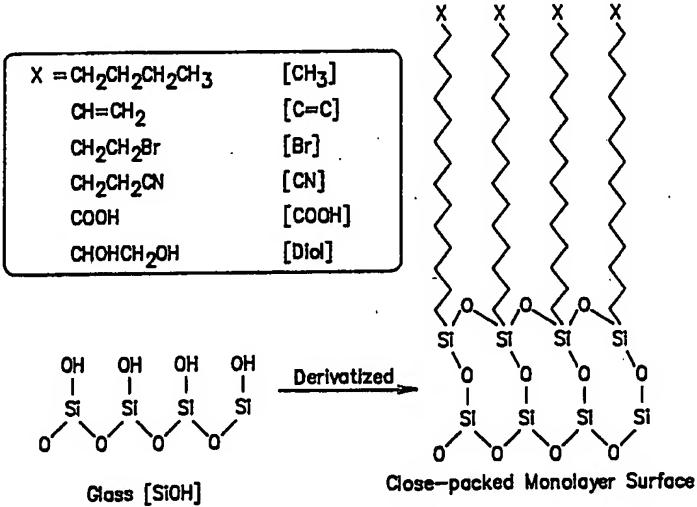
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(54) Title: PROCESS FOR CONTROLLING CELL GROWTH ON SURFACES



(57) Abstract

A process for selecting the types of cells that will grow on a structure, such as an implantable device or a cell growth surface. The implantable device may have a titanium surface. The process includes attaching a molecular monolayer to the surface of the structure. The monolayer has a functional group at its distal end. The possible function groups include CH_3 , $\text{CH}=\text{CH}_2$, Br , CN , COOH , and CHOHCH_2OH . The monolayer is coated with an adhesion-mediating molecule such as fibronectin. Cells then contact the coating. The character of the functional group affects the growth characteristics of the adhering or contacting cell, independently of the nature of the underlying structure. Also disclosed is a method of preparing a metallic surface such as titanium to receive a molecular monolayer. The surface is placed in hot water ($40\text{--}50^\circ\text{C}$) for 4 hours with sonication, or in boiling water for 8 hours without sonication.

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"Process For Controlling Cell Growth On
Surfaces"

1

BACKGROUND OF THE INVENTION

2 The present invention relates in general to cell
3 growth and, in particular, to a method for controlling cell
4 growth on a surface utilizing a molecular monolayer.

5

Description of Related Art

6 It is difficult to prepare a surface of a substrate
7 or structure in such a way that a particular type of cell
8 will attach to and grow on that surface at selective advan-
9 tage. It is important to develop an ability to prepare such
10 surfaces in order to produce such things as workable and
11 effective devices for implantation into the body. If the
12 cells of the tissue in which the implantable device is
13 implanted will not grow onto the surface of the implant,
14 causing a knitting between the implantable device and the
15 body, problems can develop with the implant and the implanta-
16 tion may fail. For example, an implantable device with a
17 porous surface is described in U.S. Patent No. 3,855,638,
18 which is incorporated herein by reference. A metallic
19 implantable device, such as a hip implant with its surface
20 covered with tiny projections or posts for tissue ingrowth,
21 is described in U.S. Patent No. 4,608,052, which is also
22 incorporated herein by reference. However, these techniques
23 suffer from the fact that the body may recognize the metal
24 as a foreign material and produce a fibrous layer between the
25 body and the implant, preventing a close knit between the
26 body and the implant.

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1 Another effort to deal with this problem has been
2 to coat the implant with a bone-like calcium phosphate
3 crystal called hydroxyapatite, which the body may accept as
4 a non-foreign bone material. However, to the extent
5 hydroxyapatite is useful, it is useful only in connection
6 with bone-forming cells. It cannot be tailored to enhance
7 or repress the growth of different, specific cell types in
8 other tissues. Moreover, good adhesion of hydroxyapatite to
9 titanium metal can be a problem.

10 It is also important to be able to control the
11 growth of particular types of cells in contact with a sur-
12 face, for example, to be able to enhance the growth of one
13 type of cell and repress or inhibit the growth of another
14 type. For example, with regard to a device implanted into
15 cartilage, it would be useful to enhance the growth of fibroblasts
16 (to knit with the cartilage) and inhibit growth
17 of neuroblasts (nerve cells, which would not be useful in
18 that situation). In this example, the fibroblasts and
19 neuroblasts are the preselected cell types, and the growth
20 of each type is controlled.

21

SUMMARY OF THE INVENTION

22 In accordance with the present invention, there is
23 provided a process for selecting the types of cells that will
24 grow on a particular surface. As used in this specification
25 and the claims herein, cell growth means cell survival, cell
26 division, and/or cell differentiation. The process comprises
27 first coating the surface with a molecular monolayer and
28 providing a preselected functional group at the distal end

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1 thereof. A layer of plasma fibronectin or other adhesion-
2 mediating molecule is then coated onto the molecular monolay-
3 er. The substrate thus prepared will affect and control the
4 growth characteristics of different cell types in contact
5 with that surface. As a result, growth of certain types of
6 cells which would facilitate tissue ingrowth and knitting
7 between implant and body can be enhanced, and growth of other
8 types of cells on the surface can be repressed or inhibited.
9 A cell growth surface is a surface upon which cell growth may
10 take place, and includes a glass slide, a petri dish, a 24-
11 well dish, and an industrial bioreactor with beads, baffles
12 and/or stirrers therein. For example, in a laboratory, cell
13 culture may be grown on surfaces in a Corning Pyrex Slow
14 Speed Stirring Vessel, #26501-1L, containing therein Kontes
15 Cytocarriers. Implantable devices include devices implant-
16 able in humans as well as devices implantable in animals.
17 As used in this specification and the claims herein, adhering
18 includes both active and passive attachment.

19 The present invention finds utility (a) in the
20 field of body implants and prosthetics, particularly implant-
21 able devices made of titanium, (b) in applications involving
22 bio-repulsive surfaces for implants and moving parts of
23 prosthetics, as well as more controlled bio-adhesive surfaces
24 for the structure of the prosthetic device, and also (c) in
25 the field of cell and tissue growth, where containers and
26 laboratory dishes and glassware with preselected surface
27 characteristics can control, enhance, repress, and otherwise
28 mediate growth of preselected cell types and cultures.
29 Surface treatments that enhance the rate of cell attachment
30 and growth would be a major benefit to both research labora-
31 tories and to the scaled-up production of specific cell lines
32 and cell-derived materials. Many aspects of the foregoing
33 discussion and invention are disclosed in Lewandowska, K.,
34 Balachander, N., Sukenik, C. N., and Culp, L.A.; "Modulation

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1 of Fibronectin Adhesive Functions for Fibroblasts and Neural
2 Cells by Chemically Derivatized Substrate," Journal of
3 Cellular Physiology, 141:334-345 (1989), the contents of
4 which are hereby incorporated by reference herein in their
5 entirety.

6 Titanium is used increasingly as an implant materi-
7 al. Among other reasons, its mechanical properties are
8 closer to those of bone than are stainless steel and cobalt-
9 chromium alloys. Coatings or surface alterations that
10 promote cell attachment and regulate physiological response
11 would make titanium even more useful. Implants made of
12 metals other than titanium could also be coated using reason-
13 ing and procedures similar to those described herein to
14 control cell attachment and regulate cell-type specific
15 physiological response.

16 Thin organic, molecular monolayer films offer an
17 excellent method for the modification of surface properties.
18 A high level of molecular monolayer uniformity can usually
19 be achieved using a carbon chain at least 14 carbons long
20 excluding the functional end group. However, somewhat
21 shorter carbon chains may be successful in this application.
22 Carbon chains containing 22 carbon atoms have been success-
23 fully prepared in other applications, and it is believed that
24 carbon chains of similar length, or longer, may be used
25 herein. The carbon chain is typically polymethylene to
26 assure sufficient chain flexibility for assembly and packing.
27 However, polymethylene chains in this application can toler-
28 ate, and are meant to include, the incorporation of double
29 bonds, an aromatic ring, a limited number of hetero-atoms,
30 and/or halogenated substituents or segments.

31 Self-assembly of SiCl_3 -terminated long-chain
32 amphiphiles forms well-ordered, siloxy-anchored, crosslinked
33 monolayers, as described in U.S. Patent No. 4,539,061 to
34 Sagiv, the contents of which are hereby incorporated by

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1 reference herein. However, Sagiv does not teach any method
2 of consistently preparing a titanium surface so that it will
3 accept a molecular monolayer. A clean titanium surface,
4 unprepared in accordance with the present invention, will
5 generally not accept or bond to a molecular monolayer as
6 described in Sagiv. The present disclosure teaches a solu-
7 tion to this problem, which comprises increasing the number
8 of hydroxy groups available for reaction on the metallic
9 surface by maintaining the metallic surface in contact with
10 boiling water for a sufficient period of time, or with water
11 at a temperature of more than 40 degrees Centigrade with
12 sonication for a sufficient period of time.

13 The modification of titanium surfaces with cova-
14 lently-attached, self-assembled monolayers offers many
15 advantages. Since the coating process involves dipping the
16 surface being treated into a dilute, homogeneous solution of
17 surfactant in an organic solvent, it is versatile and can be
18 applied to materials and implants of almost any configura-
19 tion. Coating of already fabricated implants and prostheses
20 would thus be readily achieved. Since the monolayer film so
21 completely isolates the substratum from the outside environ-
22 ment, it also permits the creation of surfaces with specific
23 properties on various bulk materials. Finally, the ease with
24 which such surfaces can be transformed by conventional
25 organic chemistry allows the creation of surfaces with the
26 functionality needed to impart desirable chemical and physi-
27 cal properties. The stability, uniformity, and manipulabili-
28 ty of these surfaces should all combine to make them useful
29 in the design of new biomaterials.

30 It is also believed that any oxide or hydroxide-
31 bearing surface similar to glass or titanium may be expected
32 to undergo chemistry and biochemistry similar to that de-
33 scribed herein. Thus, we believe that molecular monolayers
34 may be applied to a wide range of surfaces.

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1 Various functional groups have been incorporated
2 into the surface of these very uniform molecular monolayer
3 assemblies. See Balachander, N., and Sukenik, C.N., "Func-
4 tionalized Siloxy-Anchored Monolayers With Exposed Amino,
5 Azido, or Cyano Groups," Tetrahedron Letters, 29:5593-5594
6 (1988), the contents of which are incorporated by reference
7 herein. A surface to which is attached a molecular monolayer
8 having a preselected functional group at its distal end is
9 referred to as a "derivatized surface."

10 The ability of these monolayers to effectively
11 isolate their substrate is clear. Since not all functional
12 groups can coexist with the SiCl_3 group needed to anchor the
13 monolayer, surfactants or monolayer precursor molecules
14 containing a chemically modifiable group that can coexist
15 with the SiCl_3 group have been developed. Given these
16 materials and the stability of the siloxy-bound monolayer,
17 in situ generation of yet additional functionality can be
18 achieved.

19 Adhesion-mediating molecules include several
20 proteins that have cell-type-specific receptors for selected
21 cell populations. Fibronectin, as an extracellular matrix
22 glycoprotein, is an adhesion-mediating molecule that mediates
23 adhesion of many mesenchymal and some non-mesenchymal cells
24 to their collagen environment. This occurs by the binding
25 to fibronectin of (a) glycoprotein receptor complexes on the
26 cell surface called "integrins," as well as of (b) heparan
27 sulfate proteoglycans on the cell surface. This facilitates
28 the complete physiological response from some cells. Laminin
29 is also an adhesion-mediating molecule.

30 Study of the molecular mechanisms by which fibro-
31 nectins bind to artificial matrices and whether the composi-
32 tion of surface biomaterials can modulate the biological
33 activities of fibronectins coated thereon has been limited,

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1 in contrast to more extensive studies of the binding of other
2 blood-borne proteins, particularly fibrinogen.

3 In accordance with the present invention, it has
4 been shown that the binding of plasma fibronectin to
5 derivatized glass and titanium surfaces alters fibronectin
6 conformation such that cell growth of animal (including
7 human) cells such as mesenchymally-derived fibroblasts and
8 nervous system-derived neuroblastoma cells adhering to the
9 fibronectin is modulated in distinctive ways. These altered
10 responses are cell-type-specific; that is, fibroblast changes
11 on fibronectin-coated surfaces were different from those of
12 neuroblastoma cells. Mouse Balb/c 3T3 cells are an excellent
13 model of fibroblasts that come from many tissues of both
14 human and non-human animal species. In addition, Platt
15 neuroblastoma cells are an excellent model for the differen-
16 tiation processes of some neuron populations that come from
17 human and non-human species. The adhesion-mediating pro-
18 cesses of mouse Balb/c 3T3 cells are essentially identical
19 to those of normal (non-malignant) fibroblast cells. The
20 adhesion-mediating processes of Platt neuroblastoma cells are
21 essentially identical to those of normal (non-malignant)
22 neuron-derived cells. Accordingly, it is believed that the
23 results of these studies are applicable to normal (non-malig-
24 nant) fibroblast and neuron-derived cells that occur in the
25 body of human and non-human animal species.

26 It has also been shown that it makes no difference
27 whether the underlying surface is glass or titanium; as long
28 as the derivatized monolayer is the same, the cell response
29 will be the same. Thus, the underlying substratum cannot
30 "act at a distance" to affect receptor-dépendent responses
31 from cells. Chemical end groups that directly interface
32 bound fibronectin molecules clearly dominate cell responses.
33 Thus, it is believed that when different materials have their

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1 surface derivatized in the same way, the response from any
2 particular type of cell will be the same.

3 These and other aspects of this invention are more
4 fully described in the following specification.

5 BRIEF DESCRIPTION OF THE DRAWINGS

6 FIG. 1 illustrates a molecular model of derivatized
7 substrate;

8 FIG. 2 illustrates the binding of plasma
9 fibronectin to substrata;

10 FIG. 3 illustrates the quantitation of cell attach-
11 ment on substrata;

12 FIG. 4 illustrates the quantitation of neurites on
13 substrata;

14 FIG. 5 illustrates a molecular model of monolayer
15 functionalized surfaces on glass and on titanium; and

16 FIG. 6 illustrates the relative degree of
17 adsorption of plasma fibronectin to glass and titanium
18 surfaces.

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DESCRIPTION OF THE PREFERRED EMBODIMENT2 EXAMPLE I - Responses Are Cell-Type-Specific
3 On Derivatized Glass Surfaces.4 1. Summary

5 Plasma fibronectin was adsorbed onto glass surfaces
6 derivatized with an alkyl chain and six chemical end groups
7 interfacing with the bound plasma fibronectin. The response
8 of fibroblasts (Balb/c 3T3 cells) and human neuron-derived
9 (Platt neuroblastoma) cells adhering to the plasma fibro-
10 nectin was examined. Using new derivatization protocols, the
11 following surfaces were tested in order of increasing polar-
12 ity: [CH₃], [C=C], [Br], [CN], [Diol], [COOH], and
13 underivatized glass [SiOH]. For all substrata, plasma
14 fibronectin bound equivalently, using either a supersaturat-
15 ing amount of plasma fibronectin or a subsaturating amount
16 in competition with bovine albumin. Attachment of both cell
17 types was also equivalent on all substrata. However,
18 spreading/differentiation responses varied considerably.

19 Spreading and differentiation are characteristics
20 of cell growth and development. The reorganization and
21 formation of F-actin stress fibers in 3T3 cells is correlated
22 with cell growth.

23 While stress fibers formed effectively on plasma-
24 fibronectin-coated [SiOH] and [Br] substrata, only small
25 linear bundles of F-actin and a few thin stress fibers were
26 observed on the [COOH], [Diol], and [CN] substrata: the
27 hydrophobic substrata ([CH₃] and [C=C]) gave an intermediate
28 response. When a synthetic peptide containing the Arg-Gly-
29 Asp-Ser sequence required for integrin binding to
30 fibronectins was included in the medium as an inhibitor,
31 additional differences were noted: Stress fiber formation
32 was completely inhibited on [SiOH] but not on [Br] and stress
33 fiber formation was very sensitive to inhibition on the

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1 hydrophobic substrata, while the F-actin patterns on the [CN]
2 and [COOH] substrata were unaffected.

3 Neurite outgrowth by neuroblastoma cells is characteristic
4 of the specialized differentiation functions of
5 neuronal cells. Evaluation of neurite outgrowth by neuro-
6 blastoma cells on these substrata revealed both qualitative
7 and quantitative differences, as follows: [Diol] = [COOH]
8 > [SiOH] > [CN] = [Br] > [CH3] = [C=C]. While there was
9 poor cytoplasmic spreading and virtually no neurites formed
10 on the hydrophobic surfaces when plasma fibronectin alone was
11 adsorbed, neurite formation could be "rescued" if a mixture
12 of plasma fibronectin with an excess of bovine albumin was
13 adsorbed, demonstrating complex conformational interactions
14 between substratum-bound plasma fibronectin and adhesion-
15 inert neighboring molecules.

16 In summary, these experiments demonstrate that
17 different chemical end groups on the substratum modulate,
18 control, enhance, repress, and/or inhibit functions for cell
19 adhesion, growth, and their specialized differentiation
20 functions, principally by affecting the conformation of these
21 molecules rather than the amounts bound. Furthermore, these
22 experiments confirm multiple-receptor interactions with the
23 fibronectin molecules in cell-type-specific adhesion patterns.

25 2. Materials and Methods

26 a. Cells and growth conditions

27 Balb/c 3T3 (clone A31) cells were grown in
28 Dulbecco's modified Eagle's medium (DMEM) with 10% neonatal
29 calf serum, penicillin, and streptomycin in 10% CO₂: humidified
30 air (Lewandowska et al., J. Cell Biol., 105:1443-1454
31 (1987)). Human neuroblastoma cells (Platt), grown under the
32 same medium and conditions, are constitutive for production
33 of small neurites characteristic of neural tumor cells.

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1 (Vallen et al., J. Cell. Physiol., 135: 200-212 (1988).
2 Human neuroblastoma neurites are particularly useful in these
3 experiments and are easy to enumerate because of their long
4 linear neurite extension over surfaces. They require a
5 complex array of signals from the fibronectin in order to
6 achieve growth cone migration on the substratum.

7 For experiments, cells were detached at confluence,
8 after rinsing twice with phosphate-buffered saline (PBS),
9 using 0.5 mM EGTA in PBS at 37°C for 30 minutes (Lewandowska
10 et al., J. Cell Biol., 105:1443-1454 (1987)). After rinsing
11 twice, cells were resuspended in DMEM plus 250 ug/ml heat-
12 treated bovine serum albumin (BSA; referred to as "adhesion
13 medium").

14. b. Derivatization of Surfaces

15. Glass coverslips were derivatized via surface Si-
16. OH linkages, as previously described (Netzer and Sagiv, J.
17. Am. Chem. Soc., 105:674-676 (1983); Balachander and Sukenik,
18. Tetrahedron Lett., 29:5593-5594 (1988)), and as illustrated
19. in FIG. 1. FIG. 1 illustrates glass coverslips derivatized
20. by the attachment of a functionalized 14-carbon aliphatic
21. chain to the surface-available silicon atoms. Briefly, a
22. siloxane network covalently anchors an array of hydrocarbon
23. chains terminating with one of the end groups [X] interfacing
24. the medium. [X] is the active component in the binding
25. reactions of fibronectin. [CH₃] was obtained using octa-
26. decyltrichlorosilane and [C=C], [Br], and [CN] resulting from
27. SiCl₃-terminated compounds derived from 16-bromo-1-hexa-
28. decene. Deposition of the self-assembled monolayer films
29. was achieved by dipping glass coverslips, cleaned by an Argon
30. plasma, into 20 mM solutions of the SiCl₃ derivative in
31. dicyclohexyl for 2-5 minutes, achieving maximal derivati-
32. zation as ascertained below. [COOH] and [Diol] were obtained

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1 by KMnO₄ oxidation of [C=C] either with or without added
2 base, respectively.

3 Characterization of surfaces was based on 1)
4 wettability (contact angle measurements), 2) Fourier trans-
5 form infrared spectroscopy (samples prepared on silicon
6 prisms) in the attenuated total reflectance mode (FTIR-ATR),
7 and 3) electron spectroscopy for chemical analyses (ESCA).
8 [CH₃] and [C=C] surfaces are both hydrophobic and oleophobic
9 (advancing water contact angles > 110° for [CH₃] and > 105°
10 for [C=C]; hexadecane contact angles of 40-42°). FTIR-ATR
11 spectra show the expected polymethylene chains and (for
12 [CH₃]) the terminal CH₃ group (2,960 cm⁻¹). [CN] and [Br]
13 have water contact angles of 74° and 81°, respectively, and
14 show the expected ESCA signals for the heteroatom ([CN] N at
15 403 eV; [Br] Br at 72 eV, uncorrected for shift caused by
16 insulator substrate). [CN] on silicon ATR prisms have an
17 infrared absorption at 2,247 cm⁻¹. The [Diol] and [COOH]
18 surfaces, derived from the hydrophobic [C=C] monolayers, were
19 hydrophilic (water contact angles of 30° and 52°, respec-
20 tively).

21 c. Fibronectin and its adsorption

22 Human plasma fibronectin was purified from plasma
23 by affinity chromatography (Lewandowska et al., J. Cell Biol.
24 105:1443-1454 (1987) and stored in CAPS buffer at -80°C. For
25 adsorption to surfaces, plasma fibronectin was diluted to 20
26 ug/ml in PBS, and 500 ul of this solution was added to each
27 well of 24-well cluster dishes containing derivatized glass
28 coverslips for 1 hour at 37°C (Haas and Culp, J. Cell.
29 Physiol., 113:289-297 (1982); Haas et al., J. Cell Physiol.
30 120:117-125 (1984)). For coverage of coverslips, medium
31 containing heat-treated BSA was added for 1 hour at 37°C.
32 Saturability of fibronectin binding was tested using a goat
33 polyclonal antiserum to human plasma fibronectin and an

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1 enzyme-linked immunosorption assay (ELISA) as previously
2 described (Lewandowska et al., FEBS Lett. 237:35-39 (1988)).
3 BSA was adsorbed similarly and tested by ELISA as well, using
4 a polyclonal antiserum to bovine albumin.

5 d. Adhesion Assays

6 EGTA-detached 3T3 (10×10^4) or Platt (5×10^4)
7 cells were inoculated into wells containing adhesion medium
8 and plasma fibronectin-coated, derivatized glass coverslips.
9 To quantitate attachment, cells had been previously radio-
10 labeled by incorporation of [3 H] thymidine into DNA (Lewan-
11 dowska et al., J. Cell Biol. 105:1443-1454 (1987); Mugnai et
12 al., J. Cell Biol., 106:931-943 (1988)). After 1 hour,
13 unattached cells were rinsed out, attached cells rinsed twice
14 with PBS, and attached cells solubilized with a NaOH/SDS
15 mixture for quantitation of radioactivity in a scintillation
16 counter. Standard errors of multiple determinations were
17 calculated.

18 To evaluate morphological responses, cells were
19 allowed to attach and spread for 4 hours (3T3 cells) or for
20 16 hours (Platt cells) for optimal neurite development. They
21 were then fixed with 3% glutaraldehyde for photography on a
22 Nikon Diaphot microscope using Kodak technical pan 2415 film.
23 Neurites generated with Platt cells were quantitated as
24 described previously (Waite et al., Exp. Cell Res., 169:311-
25 327 (1987); Mugnai et al., J. Cell Biol., 106:931-943
26 (1988)). For higher resolution and evaluation of neurites,
27 scanning electron microscopy was also performed (Mugnai et
28 al., J. Cell Biol. 106:931-943 (1988); Mugnai et al., Eur.
29 J. Cell Biol., 46:352-361 (1988)).

30 To evaluate microfilament networks, 3T3 cells
31 spreading for 4 hours were fixed with 3.7% formaldehyde in
32 PBS for 20 minutes and then treated as previously described
33 (Laterra et al., J. Cell Biol., 96:112-123 (1983)) to bind

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1 rhodamine-phalloidin to the F-actin-containing networks.
2 Stained coverslips were inverted into 50% glycerol:PBS and
3 evaluated in the Nikon Diaphot microscope with fluorescence
4 illumination (photographed under an x100 objective with Kodak
5 2475 recording film).

6 3. Results

7 a. Fibronectin Binding to Surfaces

8 Plasma fibronectin binding to derivatized substrate
9 (FIG. 1) was tested by ELISA. FIG. 2 illustrates plasma
10 fibronectin (pFN) binding to substrata. Wells of 24-well
11 tissue culture cluster dishes, containing glass coverslips
12 with the indicated derivatization of the coverslip, were
13 adsorbed for 1 hour at 37°C with either 20 ug/ml human plasma
14 fibronectin (stippled bars) or a mixture of 2.5 ug/ml plasma
15 fibronectin plus 17.5 ug/ml bovine serum albumin (BSA);
16 slashed bars. Wells were rinsed with PBS and coverslips were
17 transferred to another 24-well cluster dish and postadsorbed
18 with 250 ug/ml heat-treated BSA for 1 hour at 37°C. After
19 rinsing the wells with PBS, the amount of plasma fibronectin
20 bound was assayed by ELISA, using goat polyclonal antihuman
21 plasma fibronectin, as described by Lewandowska et al., FEBS
22 Lett. 237:35-39 (1988). The final ELISA reactions stopped
23 with 5 M NaOH after 1 hour were transferred to 96-well dishes
24 for assaying absorbance at 405 nm in an ELISA reader. Stan-
25 dard errors of multiple determinations are shown.

26 As shown in FIG. 2, using an excess of plasma
27 fibronectin (20 ug/ml) for saturating substrata (Hughes et
28 al., Exp. Cell. Res., 121:307-314 (1979); Haas and Culp, J.
29 Cell. Physiol., 113:289-297 (1982); Lewandowska et al., FEBS
30 Lett. 237:35-39 (1988), plasma fibronectin bound comparably
31 to all surfaces as evaluated by the Student's t test.
32 Binding was also examined when a limiting amount of plasma
33 fibronectin was competing with a large excess of albumin (a

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1 molecule that cells do not interact with). When plasma
2 fibronectin (2.5 ug/ml) was mixed with an excess of bovine
3 albumin (17.5 ug/ml) prior to adsorption of the mixture to
4 surfaces (FIG. 2), plasma fibronectin still bound effectively
5 and competitively achieved a concentration on the substratum
6 similar to that of plasma fibronectin alone, indicating the
7 effectiveness of plasma fibronectin interaction with all
8 surface end groups in competition with albumin. Two excep-
9 tions were noted with the [CH₃] and [COOH] substrata with a
10 smaller amount of plasma fibronectin bound. (However, this
11 small reduction could not be an explanation for altered cell
12 responses as shown below).

13 b. Cell Attachment To Plasma Fibronectin-Coated Surfaces

14 Attachment of thymidine-radiolabeled cells was
15 determined. When bovine albumin was adsorbed, both 3T3 and
16 Platt cells failed to attach at all, demonstrating the
17 inability of cells to interact with this protein on all
18 derivatized substrata and requiring an adhesion-promoting
19 protein to mediate physiologically compatible cell responses.

20 With regard to FIG. 3, Balb/c 3T3 fibroblasts and
21 Platt neuroblastoma cells were radiolabeled separately in
22 stock cultures by incorporation of [³H] thymidine into their
23 DNA in complete medium as described above in Materials and
24 Methods. After chasing the radiolabeled precursor for 24
25 hours, cells were detached from stock culture, washed by
26 repeated resuspension/centrifugation, and enumerated: 10 x
27 10⁴ 3T3 cells or 5 x 10⁴ Platt cells were inoculated into 24-
28 well dishes containing derivatized glass coverslips coated
29 with 20 ug/ml plasma fibronectin and adhesion medium. After
30 1 hour to permit maximal attachment, unattached cells were
31 rinsed out and the adherent cells solubilized in NaOH/SDS as
32 described by Mugnai et al., J. Cell Biol., 106:931-943
33 (1988), for determination of radioactivity by scintillation

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1 counting. An equivalent sized aliquot of the cell suspension
2 was also assayed for radioactivity to determine the percent-
3 age of radiolabeled cells adherent. In FIG. 3, standard
4 errors are shown for multiple determinations.

5 As shown in FIG. 3A, when attachment had plateaued,
6 3T3 cells attached equivalently to all derivatized substrata
7 coated with plasma fibronectin. The same was true for
8 neuroblastoma cells (FIG. 3B). This contrasted with the
9 ability of both 3T3 and Platt cells to attach to all substra-
10 ta in the absence of any adsorbed plasma fibronectin or any
11 other protein (data not shown); however, cells adherent to
12 naked surfaces failed to respond further and detached within
13 6-12 hours in all cases, demonstrating the physiological
14 incompatibility of all surfaces without an appropriate
15 adhesion-promoting protein (Grinnell, Int. Rev. Cytol. 53:65-
16 144 (1978)).

17 c. Spreading and Cytoskeletal Responses of 3T3 Cells

18 In contrast to the equivalency of attachment,
19 cytoplasmic spreading and differentiation of cells were
20 significantly different among substrata. Reorganization of
21 microfilaments (F-actin) into stress fibers by fibroblasts
22 on fibronectin requires complex reactions, including trans-
23 membrane signaling from fibronectin to both heparan sulfate
24 proteoglycans (Laterra et al., J. Cell Biol. 96:112-123
25 (1983) and the glycoprotein integrin (Tamkun et al., Cell,
26 46:271-282 (1986) on the cell surface. Burridge et al.,
27 Annu. Rev. Cell Biol. 4:487-525 (1988). Therefore, stress
28 fibers and focal contacts on the substratum are a diagnostic
29 indicator of the complete response of fibroblasts permitting
30 subsequent movement, cell division, and expression of genes
31 linked to anchorage dependence (Dike and Farmer, Proc. Natl.
32 Acad. Sci. U.S.A., 85:6792-6796 (1988)).

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With regard to F-actin cytoskeletal reorganization in adherent 3T3 cells, 3T3 cells were detached from stock cultures and washed by repeated resuspension/centrifugation. Cells (7.5×10^4) were inoculated into 24-well dishes containing derivatized glass coverslips coated with 20 ug/ml plasma fibronectin and adhesion medium; they were permitted to spread and to reorganize cytoskeletal networks for 4 hours as described previously (Laterra et al., J. Cell Biol., 96:112-123 (1983); Hall et al., Exp. Cell Res., 179:115-136 (1988)). The adhesion medium and unattached cells were rinsed out of wells that were rinsed three times with PBS prior to paraformaldehyde fixation and staining of cells with rhodamine-phalloidin as described in Materials and Methods. Coverslips were photographed under epifluorescence illumination for the same exposure times on a Nikon Diaphot microscope using Kodak 2475 recording film. All negatives were also processed identically to allow direct visual comparisons among the samples; in some cases (e.g., samples [SiOH] and [Br]), this resulted in overexposed images in order to visualize samples with much poorer organization (e.g., samples [COOH] and [CN]). F-actin stress fibers formed extensively in cells on plasma fibronectin-coated glass. Thinner and shorter F-actin bundles were principally observed on the plasma fibronectin-coated carboxy substratum, while a small subset of cells therein appeared to contain some thin stress fibers. On the cyano substratum coated with plasma fibronectin, very short F-actin bundles could be observed in poorly spread cells, with some modest thin stress fibers evident at the periphery of better-spread cells. On the hydrophobic substratum represented by the methyl end group coated with plasma fibronectin, thicker stress fibers were observed in many cells and some cells had formed extensive stress fibers. The plasma fibronectin-coated bromo substratum gave cells with extensive stress fiber arrays virtually

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1 identical to the control glass surface, while the olefin
2 hydrophobic surface gave some cells with excellent stress
3 fibers.

4 As previously mentioned, Balb/c 3T3 cells were
5 examined at a 4-hour time point when cytoplasmic spreading
6 had optimized. Rhodamine-phalloidin stains extensive stress
7 fibers formed on plasma fibronectin-coated [SiOH]. In
8 contrast, cells on highly polar [COOH] and [Diol] surfaces
9 had greatly reduced F-actin organization with linear bundles
10 of limited distance and lacking the extensive pattern shown
11 with respect to [SiOH]. A similar pattern was observed on
12 [CN] surfaces. Some thin fibers could be identified in
13 approximately one-third of the cells on both [COOH] and [CN]
14 substrata. Hydrophobic substrata represented by [CH₃]
15 yielded a stress fiber pattern similar to the [SiOH] control
16 in one subpopulation of cells and some thinner fibers evident
17 in a second subpopulation of cells. The [Br] response was
18 virtually indistinguishable from [SiOH] with thick stress
19 fibers evident throughout. The [C-C] response was very
20 similar to the [CH₃] response, demonstrating consistency for
21 the two hydrophobic surfaces. These analyses indicate that
22 transmembrane signaling processes from fibronectin on these
23 surfaces are significantly different. The most polar sur-
24 faces represented by the [COOH], [Diol], and [CN] gave the
25 poorest F-actin responses; the hydrophobic surfaces of [CH₃]
26 and [C-C] an intermediate response; and the [Br] surface a
27 pattern virtually indistinguishable from the control [SiOH].
28 The most reasonable explanation, considering the comparable
29 amounts of plasma fibronectin bound to all surfaces, is
30 differing conformations of the plasma fibronectin leading to
31 differing interactions with multiple cell surface receptors
32 (see below as well). These patterns remained unchanged at
33 time points up to 24 hours, demonstrating the stability of
34 cytoskeletal reorganization.

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1 F-actin reorganization was also examined with a
2 complementary approach involving use of an inhibitor. The
3 discussion with respect thereto is contained in Lewandowska
4 et al., Journal of Cellular Physiology, 141:334-345 at 337-
5 340, 342-44 (1989), the contents of which are incorporated
6 herein by reference in their entirety. The results of the
7 inhibitor study confirmed the differing binding relationships
8 of plasma fibronectin on derivatized substrata with the
9 integrin complex as the cell surface. Since cells were
10 treated uniformly in this paradigm, these results support the
11 belief that plasma fibronectin on these substrata has differ-
12 ing conformations with varying interactions with cell surface
13 receptors (such as integrins and heparan sulfate proteo-
14 glycans).

15 d. Spreading and Neurite Outgrowth of Neural Cells
16 Cells derived from the neural crest of the embryo
17 can extend neurites on fibronectin in many cases (Rovasio et
18 al., J. Cell Biol., 96:462-472 (1983)). There are several
19 binding domains in fibronectin that may regulate this neur-
20 itogenesis (Mugnai et al., J. Cell Biol., 106:931-943
21 (1988)). Therefore, neuritogenesis was tested on derivatized
22 substrata to determine whether fibronectin conformational
23 changes generate all-or-none or intermediate responses from
24 such cells.

25 With regard to neuritogenesis of Platt neuroblasto-
26 ma cells on substrata, Platt human neuroblastoma cells were
27 detached from stock cultures by EGTA treatment, as described
28 in Materials and Methods. After washing the cells, 5×10^4
29 cells were inoculated into 24-well dishes containing deriva-
30 tized glass coverslips coated with 20 ug/ml plasma fibro-
31 nectin and adhesion medium. Cells were allowed to develop
32 neurite processes over an 18-hour period. Adherent cells
33 were fixed with glutaraldehyde and photographed under phase

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1 contrast microscopy using Kodak film on a Nikon Diaphot
2 microscope. Thin extending neurites were very evident in the
3 cells on plasma fibronectin-coated glass. Neurites were even
4 more evident in a higher percentage of cells on the plasma
5 fibronectin-coated diol substratum. In contrast, cells on
6 the plasma fibronectin-coated cyano substratum spread poorly
7 and, for the most part, failed to extend neurites. On the
8 plasma fibronectin-coated methyl substratum, thicker pro-
9 cesses were extending over the substratum from some cells;
10 and this was also the case with the olefin surface. On the
11 plasma fibronectin-coated bromo substratum, a small percent-
12 age of cells were extending along thin neurites, while the
13 majority of the cells were not extending processes.

14 Thus, human Platt neuroblastoma cells responded to
15 plasma fibronectin-coated [SiOH] in an overnight incubation
16 by spreading in a bipolar fashion; a sizable percentage of
17 cells extended neurites. On [Diol] and [COOH] substrata,
18 these cells displayed identical patterns, with neurites
19 evident in many calls. In contrast, cells on [CN] had
20 greater spreading with few neurites. The hydrophobic sur-
21 face, [CH₃], gave an intermediate response with more effec-
22 tive spreading, more bipolarity, and some thicker but shorter
23 neurites. [Br] yielded a response identical to [CN], while
24 [C-C] was virtually identical to [CH₃]. These results
25 confirm that conformational differences of plasma fibronectin
26 bound to these substrata lead to very different spreading and
27 differentiation patterns of cells adhering thereto. Further-
28 more, neural cell responses were different from those re-
29 ported above for 3T3, demonstrating that neural cells and
30 fibroblasts rely on different binding activities of plasma
31 fibronectin on substrata to achieve their respective pheno-
32 types.

33 Neurites were then quantitated (Mugnai et al., J.
34 Cell Biol., 106:931-943 (1988)). Non-neural 3T3 cells give

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1 a "background" level of process extension in this assay of
2 4-5%. With regard to quantitation of neurites or substrata
3 as illustrated in FIG. 4, Platt neuroblastoma cells were
4 treated as described above with regard to neuritogenesis of
5 Platt neuroblastoma cells on substrata. Neurite-bearing
6 cells were enumerated as defined by Mugnai et al, J. Cell
7 Biol., 106:931-943 (1988) and their percentage in the total
8 adherent cell population determined. The background level
9 of neurite-bearing cells in a non-neural cell population,
10 such as Balb/c 3T3 cells evaluated under the same conditions,
11 was routinely 4-5% (Id.). The standard errors of multiple
12 determinations are shown.

13 For Platt, there are marked differences in the
14 percentage of neurite-bearing cells on these substrata, as
15 seen in FIG. 4. They are comparably low for the two hydro-
16 phobic, the bromo, and the cyano substrata, with no statisti-
17 cal differences among them. [SiOH] yielded twice as many
18 neurite-bearing cells above the 3T3 background as the hydro-
19 phobic ones, [COOH] three times, and [Diol] four times the
20 percentage. Therefore, the most polar substrata gave the
21 ideal conformation of plasma fibronectin for maximal differ-
22 entiation of cells via the binding of one or more cell
23 surface receptors.

24 e. Albumin "Rescue" of Fibronectin
25 Functions on Hydrophobic Surfaces

26 Since hydrophobic substrata gave the poorest
27 neurite responses from Platt cells, an experiment was de-
28 signed in which plasma fibronectin would be present on the
29 substratum along with a second neutral protein, i.e., a
30 protein that had effective hydrophobic interactions such as
31 serum albumin. Therefore, [C=C] derivatized coverslips were
32 coated with either 20 ug/ml plasma fibronectin, or with a
33 mixture of 2.5 ug/ml plasma fibronectin and 17.5 ug/ml BSA

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1 (see Materials and Methods), in which plasma fibronectin
2 binding had been tested earlier by ELISA (see FIG. 2). Platt
3 neuroblastoma cells (5×10^4) were inoculated into all wells
4 containing adhesion medium and incubated 18 hours to permit
5 neurite extension. The plasma fibronectin-only coating
6 yielded poor spreading for most cells; only a rare cell
7 formed neurites. In contrast, on plasma fibronectin, BSA
8 substrata cells responded more effectively by spreading and
9 becoming bipolar; many cells now generated neurites. The
10 same results were obtained for $[\text{CH}_3]$. Therefore, the confor-
11 mational alterations of plasma fibronectins alone on the
12 hydrophobic substrata can be "reversed" by neighboring
13 interactions with "adhesion-neutral" and hydrophobic proteins
14 bound to the same surface. Conformation of plasma fibro-
15 nectins is determined not only by interactions with the
16 chemical end groups on inert substrata but also by interac-
17 tions with neighboring proteins bound to the same surface.

18 4. Discussion

19 These results provide evidence that fibronectin
20 functions can be modulated by chemical end groups of the
21 inert substratum to which fibronectin is bound. Substrata
22 of all six chemical groups adsorbed the same amounts of
23 fibronectin when compared with underivatized glass, including
24 a case in which plasma fibronectin competes with an excess
25 of albumin for binding (i.e., an 8:1 mass excess and a 28:1
26 molar excess). Since a diverse series of end groups were
27 used, the binding of fibronectin must occur through a multi-
28 plicity of amino acid side-chain interactions with substrata,
29 including hydrogen bonding, van der Waals interaction, and
30 ionic interactions, any one of which may be sufficient for
31 binding. Furthermore, these studies demonstrate that fibro-
32 nectin binding and saturation levels are independent of the

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1 polarity of substrata end groups and that fibronectin func-
2 tion for the two cell types is altered in different ways,
3 again not stringently linked to polarity. Although attach-
4 ment of cells was observed on substrata in the absence of any
5 adsorbed protein, cells failed to spread, detachment oc-
6 curred, and cell death was noted in all cases, demonstrating
7 the necessity for an adhesion-promoting protein such as
8 plasma fibronectin to facilitate physiologically compatible
9 responses from cells.

10 With either fibroblasts or neural cells, attachment
11 levels were equivalent on all six derivatized substrata when
12 compared with underivatized glass. Cell surfaces harbor
13 several classes of molecules that can mediate binding to
14 fibronectin substrata to facilitate attachment processes only
15 (including the glycoprotein integrin class, the heparan
16 sulfate proteoglycans, and the highly sialylated
17 gangliosides). These data indicate that at least one of the
18 binding domains along substratum-bound fibronectin molecules
19 is available for interactions with one or more of these
20 surface molecules in all cases.

21 However, cytoplasmic spreading and differentiation
22 require transmembrane signaling from surface receptors that
23 bind coordinately to fibronectin and to cytosolic elements
24 within the cell. In both the 3T3 and neuroblastoma systems,
25 chemically derivatized substrata modulate the functions of
26 fibronectins by altering their conformation, and thereby
27 their interactions, with the panel of cell surface "recep-
28 tors." This evidence can be summarized as follows (Table 1).

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1

TABLE 1

2

Summary of Cell Responses to Different
3 Plasma Fibronectin-Coated Derivatized Surfaces

4

5

6

7 Hydrophilic

8 [Diol]

+

+++

9 [SiOH]

+++

++

10 [COOH]

+

+++

11 [CN]

+

+

12 [Br]

+++

+

13 Hydrophobic

14 [C=C]

++

+

15 [CH₃]

++

+

16

17 1 + Only thin fibers observed;
18 + + Both thin and thick fibers present;
19 + + + Only thick fibers observed.20 2 + + + High neurite counts;
21 + + Moderate neurite counts;
22 + Low neurite counts.

23

24

25

26 First, reorganization of F-actin into stress fibers
 27 in 3T3 cells varies significantly among the seven substrata
 28 (Table 1). [SiOH] and [Br] substrata provided optimal stress
 29 fiber formation throughout the cytoplasm of all cells. The
 30 hydrophobic surfaces ([CH₃] and [C=C]) gave an intermediate
 31 response with both thick and some very thin stress fibers.
 32 The polar surfaces ([CN], [COOH], and [Diol]) gave the
 33 poorest response, with numerous star-shaped clusters of F-
 34 actin in the cytoplasm, some short linear bundles of F-actin,
 35 and, in a few cases, some very thin stress fibers.

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1 Second, the pattern of neurite formation in neuro-
2 blastoma cells was quite different from the patterns of
3 stress fiber formation in 3T3 cells (Table 1). The quantita-
4 tive and qualitative evaluations of neurites can be placed
5 into the following array: [Diol] = [COOH] > [SiOH] > > [CN]
6 = [Br] > [CH₃] = [C-C]. Therefore, cell types of very
7 different embryological origin, i.e., fibroblasts as repre-
8 sented by the model 3T3 cell and neuronal cells represented
9 by the derivative neuroblastoma cell, reacted with substrata
10 in cell type-specific ways. F-actin reorganization in the
11 3T3 cells was reasonably effective on the hydrophobic sub-
12 strata, while these substrata were the poorest for neurite
13 formation of neuroblastoma cells. The [Br] substratum
14 yielded an excellent stress fiber pattern in 3T3 cells, but
15 was poor for eliciting neurites from the neuroblastoma cells.

16 These data indicate that the conformation of
17 fibronectin molecules can be highly variable on chemically
18 derivatized substrata, and that subsequent interactions with
19 multiple cell surface receptors are affected. Cell surface
20 receptors probably interact synergistically with multiple
21 binding domains on the intact plasma fibronectin. Since only
22 intact plasma fibronectin was tested in these studies and
23 considerable differences in F-actin reorganization were
24 documented, it appears that the topology of binding domains
25 in fibronectins is critical for maximal cell surface response
26 during adhesion.

27 Of significance as well was the demonstration of
28 the "rescue" of defective fibronectin function by heterolo-
29 gous neighboring proteins on the substratum. In the present
30 experiments, hydrophobic substrata generated fibronectin
31 conformations, when this was the only protein bound, extreme-
32 ly ineffective for neurite formation by neural cells. Howev-
33 er, albumin molecules along with plasma fibronectin on these
34 substrata reverted the conformation of plasma fibronectin

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1 such that receptor interaction (therefore, neurite extension).
2 was maximal. This system will be useful for resolving the
3 fibronectin-dependent binding mechanisms critical in neurite
4 differentiation in various neuronal populations and for
5 identifying the cell receptors involved.

6 The dimeric fibronectin molecule exhibits complex
7 binding properties as it interacts with inert substrata
8 containing various end groups. These end groups can modulate
9 the functions of fibronectins during their reaction with a
10 multiplicity of cell surface receptors. This level of
11 regulation and control of adhesion-promoting proteins and the
12 cells adhering thereto is important with regard to the
13 effectiveness of biomaterial interactions with differing
14 biological systems, such as implants in a body. In this
15 regard, differing cell and tissue types from the body or
16 animal are predicted to respond differently, based on the
17 parameters observed in this study.

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1 EXAMPLE II - Responses Are Cell-Type-Specific on
2 Derivatized Titanium Surfaces In The
3 Same Way As On Derivatized Glass Surfaces

4 1. Summary

5 The surface of titanium has been modified by
6 covalent attachment of an organic molecular monolayer an-
7 chored by a siloxane network. The titanium surface often
8 requires enhancement prior to attachment of the monolayer.
9 This monolayer coating completely covers the metal and allows
10 controlled modification of surface properties by modification
11 of the exposed chemical end groups of the monolayer-forming
12 surfactant.

13 When glass and titanium are derivatized with the
14 same chemical end groups and coated with plasma fibronectin,
15 and preselected cell types are adhered thereto, the responses
16 are cell-type-specific, as discussed above, and are indepen-
17 dent of the character of the substrate as glass or titanium.
18 Identical surfaces are obtained on the glass and titanium;
19 only the monolayer coating interacts with the environment.
20 Surfaces bearing each of four different chemical end groups
21 were used; see FIG. 5. The $[\text{CH}_3]-$, $[\text{Br}]-$, and $[\text{CH}=\text{CH}_2]-$ termi-
22 nated monolayers were directly formed from surfactants
23 containing those groups and the $[\text{Diol}]$ surface was obtained
24 by oxidation of the $[\text{CH}=\text{CH}_2]$ monolayer. The surface was
25 completely derivatized with a stable, close-packed monolayer
26 with the indicated structure.

27 2. Materials and Methods

28 a. Solvents and Reagents

29 Dicyclohexyl (Aldrich) was vacuum-distilled and
30 passed through Activity I alumina (3% water by weight).
31 Doubly distilled water was used. Hexadecane was passed
32 through Al_2O_3 to remove polar contaminants. Octadecyl
33 trichlorosilane (Aldrich) was vacuum-distilled before use to

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1 deposit the $[\text{CH}_3]$ surfaces. w-Undecenyl alcohol (Aldrich)
2 was converted into w-hexadecenyl bromide (Balachander et al.,
3 Tetrahedron Lett., 29:5593-5594 (1988)). This sixteen-carbon
4 chain with an olefin at one end and a CH_2Br unit at the other
5 is used to make w-hexadecenyl-trichlorosilane (for $[\text{C}-\text{C}]$
6 surfaces, Netzer et al., J. Am. Chem. Soc., 105:674-676
7 (1983)) or 1-bromo-16-trichlorosilyl hexadecane (for $[\text{Br}]$
8 surfaces, Balachander et al., Tetrahedron Lett., 29:5593-5594
9 (1988)).

10 b. Preparation of Solid Substrates

11 Both square (22 x 22 mm) and round (to fit 24-well
12 cluster dishes) glass slides were used. They were cleaned
13 by washing with doubly distilled water, followed by
14 Soxhletting hot CHCl_3 for 1 hour. Titanium (Ti 540, 0.004
15 gauge from Teledyne Rodney Metals, CA) was sonicated in hot
16 water ($40-50^\circ\text{C}$) for 4 hours, washed in acetone, and Soxh-
17 letted in hot chloroform for 1 hour.

18 If titanium is not treated with a technique such
19 as the hot water to enhance the oxide coating, some samples
20 will not accept or bond to a molecular monolayer as described
21 in Sagiv. Heating the titanium in water at $40-50^\circ\text{C}$ for 4
22 hours with sonication, or heating it in boiling water without
23 sonication for 8 hours, provided successful monolayer attach-
24 ment for all titanium samples tested. This procedure builds
25 up the oxide layer and hydrates the surface of the titanium,
26 resulting in an adequate concentration of $\text{Ti}-\text{OH}$ moieties on
27 the surface; see FIG. 5.

28 The substrates were dried in an oven, cleaned for
29 30 minutes in an r.f. Argon plasma (Harrick PDC-3xG Plasma
30 Cleaner), and stored in fluorocarbon containers (Fluoroware)
31 and used within 1 to 2 days.

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1 c. Monolayer Coating Solutions

2 All trichlorosilane surfactants were used as 0.02-
3 0.025 M solutions in dicyclohexyl. The surfactant was added
4 to dicyclohexyl under inert atmosphere and transferred to
5 the bench top. All surfactant solutions were used within 1
6 to 3 hours after their preparation. Monolayers were prepared
7 by holding the substrate (glass or titanium) with Teflon-
8 coated tweezers and immersing it into a 10 mL beaker contain-
9 ing the surfactant solution and a magnetic stirrer. The
10 substrate is quickly withdrawn after 2 to 15 minutes, washed
11 twice with CHCl_3 and water, and Soxhletted with hot CHCl_3 or
12 1:1 v/v CHCl_3 /EtOH for 15 minutes.

13 d. Formation of Diol Surface by Oxidation
14 of [C=C] Surface With Neutral KMnO_4

15 In a beaker was placed about 100 mg of KMnO_4 and
16 20 mL of 10% aqueous acetone. The beaker was placed in an
17 ice bath at 0°C and CO_2 bubbled through it continuously. The
18 substrate with the [C=C] monolayer was dipped into the beaker
19 and kept in the solution at 0°C for 45 minutes. The monolay-
20 er substrate was removed, dipped in a 20% solution of sodium
21 bisulfite in H_2O for about 15 seconds, washed with water,
22 dried, and Soxhletted in 1:1 CHCl_3 :EtOH for 15 minutes.
23 These plates were characterized by contact angle measurements
24 and showed none of the pH dependence reported for the acid
25 surface formed by KMnO_4 cleavage of [C=C] (Maoz and Sagiv,
26 Thin Solid Films, 132:135-151 (1985)).

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1 e. Contact Angle Measurements

2 Contact angles were determined in a Rame-Hart Model
3 100 contact angle goniometer. Advancing contact angles were
4 determined by placing a drop of H₂O or hexadecane and advanc-
5 ing the periphery of the drop by adding more liquid by the
6 syringe and withdrawing the syringe and measuring the contact
7 angle within 30 seconds. The receding contact angles were
8 measured by first withdrawing part of the liquid from the
9 drop. Measurements were done at ambient temperature and
10 reported values are the average of 4 to 6 measurements taken
11 at different points on the surface.

12 f. X-Ray Photoelectron Spectroscopy

13 XPS measurements were carried out on a PHI-Unicam
14 Perkin Elmer instrument. Analyses were done using Mg K_α
15 lines at a pressure of 10⁻⁹ torr with a take-off angle of 45
16 degrees. Survey spectra were recorded on a 1 mm spot, with
17 150 eV pass energy, 200 W electron beam power, and an acqui-
18 sition time of 7 minutes. Multiplex spectra of the individ-
19 ual elements were carried out on a 1 mm spot, with 50 eV pass
20 energy and a 30-minute acquisition time. Peak positions are
21 referenced to the C 1s peak at 285 eV.

22 g. Animal Cell Adhesion and Growth Conditions

23 Human Platt neuroblastoma cells were grown in stock
24 culture in Dulbecco's modified Eagle's medium (DMEM) supple-
25 mented with 5% newborn calf serum and antibiotics. These
26 cells make neurites constitutively in serum-containing or
27 protein-free media on plasma fibronectin-adsorbed tissue
28 culture substrata. Dulbecco's medium supplemented only with
29 250 ug/mL heat-treated bovine albumin, referred to as "adhe-
30 sion medium," is used for all animal cell adhesion experi-
31 ments. Cells were treated as described in Example I. Brief-
32 ly, stock cultures were rinsed free of medium and the cells

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1 detached with 0.5 mM EGTA in phosphate-buffered saline (PBS)
2 with gentle shaking for 30 minutes at 37°C. After three
3 rinses with adhesion medium by centrifugation/resuspension,
4 the final cell pellet was suspended into adhesion medium at
5 the required concentration.

6 h. Animal Cell Adhesion Assays

7 Derivatized glass or titanium coverslips were
8 rinsed overnight with PBS (three times) prior to their
9 placement into 24-well tissue culture cluster dishes. Wells
10 were adsorbed for 60 minutes at 37°C with 20 ug/mL human
11 plasma fibronectin (purified as described in Example I) or
12 with adhesion medium to evaluate adherence to bovine albumin-
13 coated surfaces. (In all cases, attachment of neuroblastoma
14 cells was minimal on albumin coatings.) The binding of
15 plasma fibronectin to surfaces was evaluated by ELISA assay,
16 as described in Example I, using a goat polyclonal antiserum
17 directed to human plasma fibronectin, an alkaline phosphatase-conjugated indirect antibody, and absorbance at 405 nm.
18 After a 60 minute adsorption of the wells with fibronectin,
19 they were rinsed three times with PBS and postadsorbed for
20 60 minutes with adhesion medium to guarantee coverage of all
21 surface sites with the non-adhesive albumin molecule. Platt
22 neuroblastoma cells (5×10^5) were inoculated into wells and
23 incubated for 18 hours when neurite elongation over fibro-
24 nectin-coated substrata had become maximal. (In all cases,
25 longer incubation failed to improve neuritogenesis.) Wells
26 were rinsed three times with PBS and adherent cells fixed
27 prior to evaluation by microscopy. Quantitation of Platt
28 cell attachment on derivatized glass or titanium, using
29 radiolabeled cells, as described in Example I, revealed
30 standard errors varying from $\pm 3.5\%$ to $\pm 5.5\%$ for multiple
31 determinations.
32

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1 Phase contrast microscopy required 5% glutaralde-
2 hyde fixation (in PBS) of cells and examination of glass
3 coverslips under a Nikon Diaphot microscope using Kodak 2415
4 film. Cells fixed on titanium were examined and photographed
5 under epi-illumination in a Zeiss photo-microscope, using
6 the same film. For scanning electron microscopy (SEM),
7 coverslips were treated as described in Example I. Briefly,
8 they were fixed in a 2% paraformaldehyde/2% glutaraldehyde
9 mixture in 2X DMEM, dehydrated with increasing concentrations
10 of absolute ethanol-water, critical point-dried in liquid
11 CO₂, and sputter-coated with gold-palladium (Technics Hummer
12 V). Coverslips were examined on a JEOL 840 SEM (tilt angle
13 35 degrees) and photographed with Polaroid 55 positive-
14 negative film.

15 3. Results

16 a. Surface Properties

17 The monolayer coatings were initially characterized
18 by contact angle measurements and by X-ray photoelectron
19 spectroscopy (XPS). The contact angles for all the surfaces
20 used in this work are given in Table 2.

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TABLE 2
WATER CONTACT ANGLES (SESSILE DROP METHOD)

3

Water Contact Angles

4

<u>Monolayer/Substratum</u>	<u>Advancing</u>	<u>Receding</u>
[CH ₃]/Glass	112	107 - 108
[CH ₃]/Ti	110 - 112	100 - 102
[C=C]/Glass	105	98 - 100
[C=C]/Ti	103 - 106	93 - 96
[Br]/Glass	82	77 - 78
[Br]/Ti	81 - 83	65 - 70
[Diol]/Glass	30 - 34	< 10
[Diol]/Ti	26 - 35	< 10
/Bare Glass	30 - 35	10 - 15
/Bare Ti	40 - 45	10 - 15

15

16 The difference between advancing and receding
 17 contact angles (hysteresis) for a given surface is related,
 18 among other things, to the heterogeneity of the surface. The
 19 [CH₃] and [CH=CH₂] surfaces (FIG. 5) have advancing water
 20 contact angles of 110° and 105°, respectively, and are both
 21 hydrophobic and oleophobic. One or the other of these
 22 surfaces served as the hydrophobic test surface in each of
 23 the experiments below. The diol-terminated monolayer and the
 24 bare glass and titanium are all hydrophilic. The [Br]
 25 surface has an advancing contact angle of 82 degrees for both
 26 substrates, and is of intermediate hydrophobicity. The
 27 somewhat greater spread in contact angle values and the
 28 greater hysteresis for the titanium surfaces reflects greater
 29 surface heterogeneity and is consistent with the difference
 30 in texture and surface roughness seen in the scanning elec-

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1 tron microscopy (SEM) of these surfaces in the animal cell
2 adhesion study (vide infra).

3 XPS was used to verify the elemental composition
4 of the monolayer. Their hydrocarbon packing was determined
5 using the integrated intensities of the C 1s peak. Since
6 glass is insulating, the peak positions were adjusted by
7 fixing the C 1s peak at 285 eV. All surfaces showed the ex-
8 pected carbon peak and the [Br] surfaces showed the ex-
9 pected peak at 70.2 eV. The integrated peak intensities were
10 consistent with comparable monolayer packing on both glass
11 and titanium substrata and comparable packing density among
12 the various monolayers.

13 b. Animal Cell Adhesion

14 In order to evaluate human Platt cell adhesion
15 responses to titanium surfaces, it was important to establish
16 the degree of plasma fibronectin binding to both underiva-
17 tized and derivatized surfaces. This was done using an ELISA
18 assay. With respect to FIG. 6, glass or titanium coverslips,
19 either underivatized (Non deriv.) or derivatized as indi-
20 cated, were adsorbed with plasma fibronectin at a concentra-
21 tion of 20 ug/mL or with 250 ug/mL bovine albumin on underiv-
22 atized surfaces (BSA) as defined in Materials and Methods.
23 After 1 hour of adsorption, coverslips in wells were rinsed
24 with PBS and adsorbed fibronectin tested in an ELISA assay
25 as described in Materials and Methods. Standard errors of
26 multiple determinations are shown with the error bars.

27 As shown in FIG. 6, where a super-saturating amount
28 of plasma fibronectin (20 ug/mL) was incubated with either
29 glass or titanium coverslips for 1 hour, comparable amounts
30 of fibronectin bound to both underivatized glass or titanium
31 surfaces, as well as to the three classes of derivatized
32 glass or titanium surfaces. As expected, albumin adsorption
33 blocked the substratum from plasma fibronectin binding and

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1 provided a negative control substratum to evaluate cell
2 responses. These results indicate that alteration in cell
3 responses cannot be ascribed to reduced levels of plasma
4 fibronectin binding to these artificial surfaces, since
5 fibronectin can be diluted on the substratum with bovine
6 albumin to less than 5% of the concentrations displayed here
7 and still yield a maximal adhesion response (Hughes et al.,
8 Exp. Cell Res., 121:307-314 (1979); Haas and Culp, J. Cell.
9 Physiol., 113:289-297 (1982)).

10 Platt neuroblastoma cells were then inoculated onto
11 various glass or titanium coverslips adsorbed with plasma
12 fibronectin and incubated 18 hours to allow stabilization of
13 adhesion responses and maximal neurite outgrowth. Since
14 these neuronal cells require several different receptors to
15 interact with different binding domains of fibronectin, this
16 cell system is particularly sensitive to conformational
17 changes that may occur upon fibronectin binding to various
18 derivatized substrata. Cell attachment on all surfaces was
19 comparable, but spreading and neurite responses mediated by
20 transmembrane signaling processes were quite different. On
21 underivatized glass and titanium, neuroblastoma cells became
22 bipolar; some cells were extending, short, neurite-like pro-
23 cesses, while some cells were extending, long, linear, thin
24 neurites. On [Diol] surfaces of either glass or titanium,
25 the responses were different from the underivatized controls,
26 but similar to each other, i.e., a higher percentage of cells
27 were extending, long, linear, thin neurites, indicating that
28 growth cone migration over the substratum was facilitated on
29 this particular surface whether it was on glass or titanium.
30 Similarly, responses on the [Br] surfaces of either glass or
31 titanium were reduced. Cell spreading was not as extensive,
32 processes were shorter, and long, thin neurites were not
33 observed. This was even more dramatic on the [C=C] surfaces

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1 on both glass and titanium, where cells spread in a pseudo-
2 podial fashion, much like that of fibroblasts, and not of
3 neural cells; and, in addition, neurite processes in bipolar
4 cells were rarely evident. These results indicate that
5 derivatization of glass and titanium yields similar differ-
6 ences in neural cell response on these plasma fibronectin-
7 coated surfaces. Modulation of fibronectin adhesion re-
8 sponds is end-group-specific and independent of the underly-
9 ing glass or titanium. When these surfaces were tested for
10 cell responses in the absence of any adsorbed protein, cells
11 attached to all surfaces for several hours without any cyto-
12 plasmic spreading, became metabolically unbalanced, and
13 detached as dead cells. This indicates the significance of
14 adsorption of an adhesion-promoting protein, such as plasma
15 fibronectin, for physiologically-compatible cell responses.

16 These morphological changes in neuroblastoma cell
17 responses were further documented in the scanning electron
18 microscope (SEM). With regard to the scanning electron
19 microscope, neuroblastoma cells were inoculated onto underi-
20 vativized, [Diol]-derivatized, or [C=C]-derivatized titanium
21 coverslips as described in Materials and Methods. The etched
22 surface of titanium coverslips was readily apparent in all
23 cases, and was independent of the derivatization process
24 being used. A notable feature in the SEM images of the
25 titanium surfaces was their etched appearance, whether they
26 were derivatized or not, and whether they were adsorbed with
27 plasma fibronectin or not. This indicates ultrastructural
28 differences in the metallic surface in contrast to the smooth
29 appearance of all glass surfaces in Example I. With respect
30 to underivatized titanium coated with plasma fibronectin, the
31 most common neural cell response was a bipolar cell extending
32 two thickened neurite-like processes at both ends, and with
33 migrating growth cones at the ends of these "neurites"
34 containing actively ruffling plasma membrane. In contrast,

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1 on the [Diol] titanium surface where responses were excel-
2 lent, long and thin neurites were readily evident in two
3 highly bipolar cells, and these neurites could extend >100
4 μ m from the cell bodies of cells. These excellent neurites
5 were observed in a smaller proportion of cells on the underi-
6 vated surfaces, rarely on the [Br] surface, and virtually
7 not at all on the [C=C] surfaces. On this last substratum,
8 cell spreading was evident in a very different pattern:
9 broad pseudopodial processes, reminiscent of fibroblast
10 responses to fibronectin and not neural cells, were common
11 for most cells. These analyses document the cellular ultra-
12 structural changes that occur in response to similar amounts
13 of fibronectin on various derivatized surfaces, and undoubt-
14 edly reflect the differing natures of multiple cell surface
15 receptors interacting with substratum-bound fibronectin
16 molecules. These differences also occur independently of the
17 etched nature of all titanium surfaces analyzed to date,
18 since derivatization of very smooth glass surfaces yields the
19 same chemical end-group-specific changes in cell responses.

20 4. Discussion

21 First, plasma fibronectin binds comparably to
22 derivatized glass or titanium surfaces; fibronectin binding
23 is not limiting cell response. However, this binding was
24 only tested in homogeneous solutions of plasma fibronectin.

25 Second, these results with animal cell adhesion
26 responses verify that the chemical end groups facing the
27 medium, and therefore interacting directly with fibronectin
28 molecules bound to the surface, alter the conformation of
29 fibronectin molecules in ways that lead to differing cell
30 surface receptor responses from cells. Therefore derivati-
31 zation of biomaterials can be used to manipulate the short
32 term (and possibly long term) responses from select animal
33 cell types.

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1 Finally, there were no detectable differences
2 between the titanium series or the glass series, revealing
3 that the underlying substratum cannot "act at a distance" to
4 affect receptor-dependent responses from cells. Chemical end
5 groups that directly interface bound fibronectin molecules
6 clearly dominate cell responses. These results support the
7 utilization of many different biomaterials derivatized with
8 similar approaches in order to achieve the same responses
9 from cells.

10 It should be understood that various modifications,
11 changes, and replacements of the components and methods
12 herein may be resorted to by those skilled in the art without
13 departing from the scope of the invention as disclosed and
14 claimed herein.

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WHAT IS CLAIMED IS:

1 1. A process for selecting the types of cells that
2 will grow on a structure, and for controlling the growth
3 thereof, comprising the steps of:

4 (a) providing on the structure a molecular
5 monolayer of molecules having proximal and distal ends, the
6 proximal ends of said molecules being attached to the struc-
7 ture, the distal ends of said molecules being provided with
8 a functional group;

9 (b) causing a coating of an adhesion-mediating
10 molecule to be formed on said molecular monolayer for inter-
11 action with said functional group so as to provide a surface
12 which tends to control the growth of preselected cell types;
13 and

14 (c) contacting said coating of said adhesion-
15 mediating molecule with cells and tending to selectively
16 control the growth thereof.

1 2. A process according to claim 1, wherein said
2 functional group is selected from the group consisting of
3 CH_3 , $\text{CH}=\text{CH}_2$, Br , CN , COOH , and CHOHCH_2OH .

1 3. A process according to claim 1, wherein said
2 adhesion-mediating molecule is fibronectin.

1 4. A process according to claim 1, wherein the
2 structure is comprised at least in part of a material se-
3 lected from the group consisting of glass and titanium.

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1 5. A process in accordance with claim 1, including
2 selecting Br as said functional group and thereby tending to
3 enhance the growth of fibroblasts and tending to inhibit or
4 repress the growth of neuron-derived cells.

1 6. A process in accordance with claim 1, includ-
2 ing selecting one of COOH and CHOCH₂OH as said functional
3 group and thereby tending to inhibit or repress the growth
4 of fibroblasts and tending to enhance the growth of neuron-
5 derived cells.

1 7. A process in accordance with claim 1, includ-
2 ing selecting CN as said functional group and thereby tend-
3 ing to inhibit or repress the growth of fibroblasts and
4 tending to inhibit or repress the growth of neuron-derived
5 cells.

1 8. A process in accordance with claim 1, includ-
2 ing selecting one of CH=CH₂ and CH₃ as said functional group
3 and thereby tending to enhance the growth of fibroblasts and
4 tending to inhibit or repress the growth of neuron-derived
5 cells.

1 9. A process according to claim 1, wherein said
2 proximal ends of said molecules comprise SiCl₃ groups for
3 attachment to said structure and said structure includes OH
4 groups for reaction therewith and the step of providing said
5 molecular monolayer includes reacting said SiCl₃ groups with
6 said OH groups to form a binding siloxane group.

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1 10. A process according to claim 1, wherein step
2 (a) includes providing on the structure a molecular monolayer
3 of molecules having a first functional group on the distal
4 ends thereof, and chemically reacting said first functional
5 group to form a second functional group.

1 11. A process according to claim 1, wherein said
2 structure includes a titanium surface portion for attachment
3 of said molecular monolayer and the step of providing said
4 molecular monolayer on said surface portion includes pre-
5 treating said titanium surface by contacting it with boiling
6 water for a period of time sufficient to increase the
7 concentration of Ti-OH moieties to enable attachment of the
8 molecular monolayer.

1 12. A process according to claim 1, wherein said
2 structure includes a titanium surface portion for attachment
3 of said molecular monolayer and the step of providing said
4 molecular monolayer on said surface portion includes pre-
5 treating said titanium surface by contacting it with water
6 at a temperature of more than 40°C, with sonication, for a
7 period of time sufficient to increase the concentration of
8 Ti-OH moieties to enable attachment of the molecular mono-
9 layer.

1 13. A process according to claim 9, wherein said
2 functional group is selected from the group consisting of
3 CH₃, CH=CH₂, Br, CN, COOH, and CHOCH₂OH, said adhesion-
4 mediating molecule is fibronectin, and the structure is
5 comprised at least in part of a material selected from the
6 group consisting of glass and titanium.

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1 14. A method for controlling and modulating the
2 function of an adhesion-mediating molecule associated with
3 a structure in connection with the growth of cells contacting
4 the adhesion-mediating molecule, comprising the steps of:

5 (a) providing on the structure a molecular
6 monolayer of molecules having proximal and distal ends, the
7 proximal ends of said molecules being attached to the struc-
8 ture, the distal ends of said molecules being provided with
9 a functional group; and

10 (b) causing a coating of the adhesion-mediating
11 molecule to be formed on said molecular monolayer for inter-
12 action with said functional group so as to provide a surface
13 which tends to control the growth of preselected cell types
14 contacting the coating.

1 15. A method according to claim 14, wherein said
2 functional group is selected from the group consisting of
3 CH₃, CH=CH₂, Br, CN, COOH, and CHOCH₂OH.

1 16. A method according to claim 14, wherein said
2 adhesion-mediating molecule is fibronectin.

1 17. A method according to claim 14, wherein the
2 structure is comprised at least in part of a material se-
3 lected from the group consisting of glass and titanium.

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1 18. A method according to claim 14, wherein said
2 proximal ends of said molecules comprise SiCl_3 groups for
3 attachment to said structure and said structure includes OH
4 groups for reaction therewith and the step of providing said
5 molecular monolayer includes reacting said SiCl_3 groups with
6 said OH groups to form a binding siloxane group.

1 19. A method according to claim 14, wherein step
2 (a) includes providing on the structure a molecular monolayer
3 of molecules having a first functional group on the distal
4 ends thereof, and chemically reacting said first functional
5 group to form a second functional group.

1 20. A method according to claim 18, wherein said
2 functional group is selected from the group consisting of
3 CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOHCH_2OH , and said adhesion-
4 mediating molecule is fibronectin.

1 21. A method for isolating a layer of an adhesion-
2 mediating molecule from the effects of an underlying struc-
3 ture, comprising the steps of:

4 (a) providing on the structure a molecular mono-
5 layer of molecules having proximal and distal ends, the
6 proximal ends of said molecules being attached to the struc-
7 ture, substantially all molecules of said monolayer having
8 a carbon chain at least fourteen carbons long; and
9 (b) causing a coating of an adhesion-mediating
10 molecule to be formed on said monolayer.

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1 22. A method according to claim 21, wherein the
2 molecules have a functional group at their distal ends, the
3 adhesion-mediating molecules of said coating interacting with
4 said functional group to provide a surface which tends to
5 control the growth of preselected cell types contacting said
6 coating of the adhesion-mediating molecule substantially
7 independent of said underlying structure.

1 23. A method according to claim 21, wherein the
2 adhesion-mediating molecule is fibronectin.

1 24. A method according to claim 21, wherein the
2 structure is comprised at least in part of a material se-
3 lected from the group consisting of glass and titanium.

1 25. A method for isolating a cell from the effects
2 of an underlying structure, comprising the steps of:
3 (a) providing on the structure a molecular mono-
4 layer of molecules having proximal and distal ends, the
5 proximal ends of said molecules being attached to the struc-
6 ture, substantially all molecules of said monolayer having
7 a carbon chain at least fourteen carbons long;
8 (b) causing a coating of an adhesion-mediating
9 molecule to be formed on said monolayer; and
10 (c) contacting the cell with said coating.

1 26. A method according to claim 25, wherein step
2 (c) includes adhering the cell to said coating for controlled
3 cell growth in isolation from the effects of the underlying
4 structure.

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1 27. A method according to claim 25, wherein the
2 molecules have a functional group at their distal ends, the
3 adhesion-mediating molecules of said coating interacting with
4 said functional group to provide a surface which tends to
5 control the growth of the cell contacting said coating
6 substantially independent of said underlying structure.

1 28. A method according to claim 25, wherein the
2 adhesion-mediating molecule is fibronectin.

1 29. A method according to claim 25, wherein the
2 structure is comprised at least in part of a material se-
3 lected from the group consisting of glass and titanium.

1 30. A method for making an implantable device
2 including a structure portion for control of cell growth,
3 comprising the steps of:

4 (a) providing on the structure portion a molecular
5 monolayer of molecules having proximal and distal ends, the
6 proximal ends of said molecules being attached to the struc-
7 ture portion, the distal ends of said molecules being pro-
8 vided with a functional group; and

9 (b) causing a coating of an adhesion-mediating
10 molecule to be formed on said molecular monolayer for inter-
11 action with said functional group so as to provide a surface
12 which tends to control the growth of preselected cell types
13 contacting the coating.

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1 31. A method according to claim 30, wherein said
2 functional group is selected from the group consisting of
3 CH₃, CH=CH₂, Br, CN, COOH, and CHOCH₂OH.

1 32. A method according to claim 30, wherein said
2 adhesion-mediating molecule is fibronectin.

1 33. A method according to claim 30, wherein the
2 structure portion is comprised at least in part of a material
3 selected from the group consisting of glass and titanium.

1 34. A method according to claim 30, wherein said
2 proximal ends of said molecules comprise SiCl₃ groups for
3 attachment to said structure portion and said structure
4 portion includes OH groups for reaction therewith and the
5 step of providing said molecular monolayer includes reacting
6 said SiCl₃ groups with said OH groups to form a binding
7 siloxane group.

1 35. A method according to claim 34, wherein said
2 functional group is selected from the group consisting of
3 CH₃, CH=CH₂, Br, CN, COOH, and CHOCH₂OH, and said adhesion-
4 mediating molecule is fibronectin, and the structure portion
5 is comprised at least in part of a material selected from the
6 group consisting of glass and titanium.

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1 36. A method for making a cell growth surface, the
2 growth surface including a support surface, the method
3 comprising the steps of:

4 (a) providing on the support surface a molecular
5 monolayer of molecules having proximal and distal ends, the
6 proximal ends of said molecules being attached to the support
7 surface, the distal ends of said molecules being provided
8 with a functional group; and

9 (b) causing a coating of an adhesion-mediating
10 molecule to be formed on said molecular monolayer for inter-
11 action with said functional group so as to provide the growth
12 surface which tends to control the growth of preselected cell
13 types contacting the coating.

1 37. A method according to claim 36, wherein said
2 functional group is selected from the group consisting of
3 CH₃, CH=CH₂, Br, CN, COOH, and CHOCH₂OH.

1 38. A method according to claim 36, wherein said
2 adhesion-mediating molecule is fibronectin.

1 39. A method according to claim 36, wherein the
2 support surface is comprised at least in part of a material
3 selected from the group consisting of glass and titanium.

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1 40. A method according to claim 36, wherein said
2 proximal ends of said molecules comprise SiCl_3 groups for
3 attachment to said support surface and said support surface
4 includes OH groups for reaction therewith and the step of
5 providing said molecular monolayer includes reacting said
6 SiCl_3 groups with said OH groups to form a binding siloxane
7 group.

1 41. A method according to claim 40, wherein said
2 functional group is selected from the group consisting of
3 CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOHCH_2OH , and said adhesion-
4 mediating molecule is fibronectin, and the support surface
5 is comprised at least in part of a material selected from the
6 group consisting of glass and titanium.

1 42. An implantable device including a growth
2 surface having improved ability to knit itself to surrounding
3 tissue in a living organism, comprising:

4 (a) a structural portion having a support surface;
5 (b) a molecular monolayer of molecules having
6 proximal and distal ends, the proximal ends of said molecules
7 being attached to said support surface, said molecules of
8 said monolayer having a functional group at the distal ends
9 thereof; and

10 (c) a layer of an adhesion-mediating molecule
11 coating said monolayer, said functional group interacting
12 with the adhesion-mediating molecules of said layer to
13 provide the growth surface which tends to control the growth
14 of preselected cell types contacting said layer.

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1 43. An implantable device according to claim 42,
2 wherein the functional group is selected from the group
3 consisting of CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOCH_2OH .

1 44. An implantable device according to claim 42,
2 wherein the adhesion-mediating molecule is fibronectin.

1 45. An implantable device according to claim 42,
2 wherein the support surface is comprised at least in part of
3 a material selected from the group consisting of glass and
4 titanium.

1 46. An implantable device according to claim 42,
2 wherein said proximal ends of said molecules are attached to
3 said support surface by means of binding siloxane groups.

1 47. An implantable device according to claim 46,
2 wherein the functional group is selected from the group
3 consisting of CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOCH_2OH , and
4 the adhesion-mediating molecule is fibronectin, and the
5 support surface is comprised at least in part of a material
6 selected from the group consisting of glass and titanium.

1 48. A cell growth surface comprising:
2 (a) a support surface;
3 (b) a molecular monolayer of molecules having
4 proximal and distal ends, the proximal ends of said molecules
5 being attached to said support surface, said molecules of
6 said monolayer having a functional group at the distal ends

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7 thereof; and

8 (c) a layer of an adhesion-mediating molecule
9 coating said monolayer, said functional group interacting
10 with the adhesion-mediating molecules of said layer to
11 provide the growth surface which tends to control the growth
12 of preselected cell types contacting said layer.

1 49. A cell growth surface according to claim 48,
2 wherein the functional group is selected from the group
3 consisting of CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOHCH_2OH .

1 50. A cell growth surface according to claim 48,
2 wherein the adhesion-mediating molecule is fibronectin.

1 51. A cell growth surface according to claim 48,
2 wherein the support surface is comprised at least in part of
3 a material selected from the group consisting of glass and
4 titanium.

1 52. A cell growth surface according to claim 48,
2 wherein said proximal ends of said molecules are attached to
3 said support surface by means of binding siloxane groups.

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1 53. A cell growth surface according to claim 52,
2 wherein the functional group is selected from the group
3 consisting of CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOHCH_2OH , and
4 the adhesion-mediating molecule is fibronectin, and the
5 support surface is comprised at least in part of a material
6 selected from the group consisting of glass and titanium.

1 54. A process for the preparation of a metallic
2 surface of an article so as to permit formation of a molecu-
3 lar monolayer on said surface, comprising contacting said
4 metallic surface with water at an elevated temperature for
5 a period of time sufficient to increase the concentration of
6 OH moieties to enable attachment of the molecular monolayer.

1 55. A process according to claim 54, wherein the
2 metallic surface is titanium.

1 56. A process according to claim 54, wherein said
2 elevated temperature is approximately 100°C .

1 57. A process according to claim 56, wherein the
2 metallic surface is titanium.

1 58. A process according to claim 54, wherein said
2 elevated temperature is at least 40°C , and further including
3 sonicating the article.

1 59. A process according to claim 58, wherein the
2 metallic surface is titanium.

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AMENDED CLAIMS

[received by the International Bureau on 09 December 1991 (09.12.91);
original claims 1-59 replaced by amended claims 1-59
(13 pages)]

1 1. A process for selecting the types of cells that
2 will grow on a substrate, and for controlling the growth
3 thereof, comprising the steps of:

4 (a) providing on the substrate a metallic surface
5 structure and a molecular monolayer of molecules thereon
6 having proximal and distal ends, the proximal ends of said
7 molecules being attached to the structure, the distal ends
8 of said molecules being provided with a functional group;

9 (b) causing a coating of an adhesion-mediating
10 molecule to be formed on said molecular monolayer for inter-
11 action with said functional group so as to provide a surface
12 which tends to control the growth of preselected cell types;
13 and

14 (c) contacting said coating of said adhesion-
15 mediating molecule with cells and tending to selectively
16 control the growth thereof.

1 2. A process according to claim 1, wherein said
2 functional group is selected from the group consisting of
3 CH_3 , $\text{CH}=\text{CH}_2$, Br , CN , COOH , and CHOHCH_2OH .

1 3. A process according to claim 1, wherein said
2 adhesion-mediating molecule is fibronectin.

1 4. A process according to claim 1, wherein the
2 metallic surface structure is titanium.

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1 5. A process in accordance with claim 1, including
2 selecting Br as said functional group and thereby tending to
3 enhance the growth of fibroblasts and tending to inhibit or
4 repress the growth of neuron-derived cells.

1 6. A process in accordance with claim 1, includ-
2 ing selecting one of COOH and CHOCH₂OH as said functional
3 group and thereby tending to inhibit or repress the growth
4 of fibroblasts and tending to enhance the growth of neuron-
5 derived cells.

1 7. A process in accordance with claim 1, includ-
2 ing selecting CN as said functional group and thereby tend-
3 ing to inhibit or repress the growth of fibroblasts and
4 tending to inhibit or repress the growth of neuron-derived
5 cells.

1 8. A process in accordance with claim 1, includ-
2 ing selecting one of CH=CH₂ and CH₃ as said functional group
3 and thereby tending to enhance the growth of fibroblasts and
4 tending to inhibit or repress the growth of neuron-derived
5 cells.

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1 9. A process according to claim 1, wherein said
2 proximal ends of said molecules comprise SiCl_3 groups for
3 attachment to said structure and said structure includes OH
4 groups for reaction therewith and the step of providing said
5 molecular monolayer includes reacting said SiCl_3 groups with
6 said OH groups to form a binding siloxane group.

1 10. A process according to claim 1, wherein step
2 (a) includes providing on the structure a molecular monolayer
3 of molecules having a first functional group on the distal
4 ends thereof, and chemically reacting said first functional
5 group to form a second functional group.

1 11. A process according to claim 1, wherein said
2 structure includes a titanium surface portion for attachment
3 of said molecular monolayer and the step of providing said
4 molecular monolayer on said surface portion includes pre-
5 treating said titanium surface by contacting it with boiling
6 water for a period of time sufficient to increase the
7 concentration of Ti-OH moieties to enable attachment of the
8 molecular monolayer.

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1 12. A process according to claim 1, wherein said
2 structure includes a titanium surface portion for attachment
3 of said molecular monolayer and the step of providing said
4 molecular monolayer on said surface portion includes pre-
5 treating said titanium surface by contacting it with water
6 at a temperature of more than 40°C, with sonication, for a
7 period of time sufficient to increase the concentration of
8 Ti-OH moieties to enable attachment of the molecular mono-
9 layer.

1 13. A process according to claim 9, wherein said
2 functional group is selected from the group consisting of
3 CH₃, CH=CH₂, Br, CN, COOH, and CHOCH₂OH, and said adhesion-
4 mediating molecule is fibronectin.

1 14. A method for controlling and modulating the
2 function of an adhesion-mediating molecule associated with
3 a substrate in connection with the growth of cells contacting
4 the adhesion-mediating molecule, comprising the steps of:

5 (a) providing on the substrate a metallic surface
6 structure and a molecular monolayer of molecules thereon
7 having proximal and distal ends, the proximal ends of said
8 molecules being attached to the structure, the distal ends
9 of said molecules being provided with a functional group; and

10 (b) causing a coating of the adhesion-mediating
11 molecule to be formed on said molecular monolayer for inter-
12 action with said functional group so as to provide a surface
13 which tends to control the growth of preselected cell types
14 contacting the coating.

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1 15. A method according to claim 14, wherein said
2 functional group is selected from the group consisting of
3 CH₃, CH=CH₂, Br, CN, COOH, and CHOCH₂OH.

1 16. A method according to claim 14, wherein said
2 adhesion-mediating molecule is fibronectin.

1 17. A method according to claim 14, wherein the
2 structure is titanium.

1 18. A method according to claim 14, wherein said
2 proximal ends of said molecules comprise SiCl₃ groups for
3 attachment to said structure and said structure includes OH
4 groups for reaction therewith and the step of providing said
5 molecular monolayer includes reacting said SiCl₃ groups with
6 said OH groups to form a binding siloxane group.

1 19. A method according to claim 14, wherein step
2 (a) includes providing on the structure a molecular monolayer
3 of molecules having a first functional group on the distal
4 ends thereof, and chemically reacting said first functional
5 group to form a second functional group.

1 20. A method according to claim 18, wherein said
2 functional group is selected from the group consisting of
3 CH₃, CH=CH₂, Br, CN, COOH, and CHOCH₂OH, and said adhesion-
4 mediating molecule is fibronectin.

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1 21. A method for isolating a layer of an adhesion-
2 mediating molecule from the effects of an underlying struc-
3 ture, comprising the steps of:

4 (a) providing on the structure a molecular mono-
5 layer of molecules having proximal and distal ends, the
6 proximal ends of said molecules being attached to the struc-
7 ture, substantially all molecules of said monolayer having
8 a carbon chain at least fourteen carbons long; and

9 (b) causing a coating of an adhesion-mediating
10 molecule to be formed on said monolayer.

1 22. A method according to claim 21, wherein the
2 molecules have a functional group at their distal ends, the
3 adhesion-mediating molecules of said coating interacting with
4 said functional group to provide a surface which tends to
5 control the growth of preselected cell types contacting said
6 coating of the adhesion-mediating molecule substantially
7 independent of said underlying structure.

1 23. A method according to claim 21, wherein the
2 adhesion-mediating molecule is fibronectin.

1 24. A method according to claim 21, wherein the
2 structure is comprised at least in part of a material se-
3 lected from the group consisting of glass and titanium.

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1 25. A method for isolating a cell from the effects
2 of an underlying structure, comprising the steps of:

3 (a) providing on the structure a molecular mono-
4 layer of molecules having proximal and distal ends, the
5 proximal ends of said molecules being attached to the struc-
6 ture, substantially all molecules of said monolayer having
7 a carbon chain at least fourteen carbons long;

8 (b) causing a coating of an adhesion-mediating
9 molecule to be formed on said monolayer; and

10 (c) contacting the cell with said coating.

1 26. A method according to claim 25, wherein step
2 (c) includes adhering the cell to said coating for controlled
3 cell growth in isolation from the effects of the underlying
4 structure.

1 27. A method according to claim 25, wherein the
2 molecules have a functional group at their distal ends, the
3 adhesion-mediating molecules of said coating interacting with
4 said functional group to provide a surface which tends to
5 control the growth of the cell contacting said coating
6 substantially independent of said underlying structure.

1 28. A method according to claim 25, wherein the
2 adhesion-mediating molecule is fibronectin.

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1 29. A method according to claim 25, wherein the
2 structure is comprised at least in part of a material se-
3 lected from the group consisting of glass and titanium.

1 30. A method for making an implantable device
2 including a structure portion for control of cell growth,
3 comprising the steps of:

4 (a) providing on the structure portion a molecular
5 monolayer of molecules having proximal and distal ends, the
6 proximal ends of said molecules being attached to the struc-
7 ture portion, the distal ends of said molecules being pro-
8 vided with a functional group; and

9 (b) causing a coating of an adhesion-mediating
10 molecule to be formed on said molecular monolayer for inter-
11 action with said functional group so as to provide a surface
12 which tends to control the growth of preselected cell types
13 contacting the coating.

1 31. A method according to claim 30, wherein said
2 functional group is selected from the group consisting of
3 CH_3 , $\text{CH}=\text{CH}_2$, Br , CN , COOH , and CHOHCH_2OH .

1 32. A method according to claim 30, wherein said
2 adhesion-mediating molecule is fibronectin.

1 33. A method according to claim 30, wherein the
2 structure portion is titanium.

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1 34. A method according to claim 30, wherein said
2 proximal ends of said molecules comprise SiCl_3 groups for
3 attachment to said structure portion and said structure
4 portion includes OH groups for reaction therewith and the
5 step of providing said molecular monolayer includes reacting
6 said SiCl_3 groups with said OH groups to form a binding
7 siloxane group.

1 35. A method according to claim 34, wherein said
2 functional group is selected from the group consisting of
3 CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOCH_2OH , and said adhesion-
4 mediating molecule is fibronectin, and the structure portion
5 is titanium.

1 36. A method for making a cell growth surface, the
2 growth surface including a substrate, the method comprising
3 the steps of:

4 (a) providing on the substrate a metallic support
5 surface and a molecular monolayer of molecules thereon having
6 proximal and distal ends, the proximal ends of said molecules
7 being attached to the support surface, the distal ends of
8 said molecules being provided with a functional group; and

9 (b) causing a coating of an adhesion-mediating
10 molecule to be formed on said molecular monolayer for inter-
11 action with said functional group so as to provide the growth
12 surface which tends to control the growth of preselected cell
13 types contacting the coating.

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1 37. A method according to claim 36, wherein said
2 functional group is selected from the group consisting of
3 CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOHCH_2OH .

1 38. A method according to claim 36, wherein said
2 adhesion-mediating molecule is fibronectin.

1 39. A method according to claim 36, wherein the
2 metallic support surface is titanium.

1 40. A method according to claim 36, wherein said
2 proximal ends of said molecules comprise SiCl_3 groups for
3 attachment to said support surface and said support surface
4 includes OH groups for reaction therewith and the step of
5 providing said molecular monolayer includes reacting said
6 SiCl_3 groups with said OH groups to form a binding siloxane
7 group.

1 41. A method according to claim 40, wherein said
2 functional group is selected from the group consisting of
3 CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOHCH_2OH , and said adhesion-
4 mediating molecule is fibronectin.

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1 42. An implantable device including a growth
2 surface having improved ability to knit itself to surrounding
3 tissue in a living organism, comprising:

4 (a) a structural portion having a support surface;

5 (b) a molecular monolayer of molecules having
6 proximal and distal ends, the proximal ends of said molecules
7 being attached to said support surface, said molecules of
8 said monolayer having a functional group at the distal ends
9 thereof; and

10 (c) a layer of an adhesion-mediating molecule
11 coating said monolayer, said functional group interacting
12 with the adhesion-mediating molecules of said layer to
13 provide the growth surface which tends to control the growth
14 of preselected cell types contacting said layer.

1 43. An implantable device according to claim 42,
2 wherein the functional group is selected from the group
3 consisting of CH_3 , $\text{CH}=\text{CH}_2$, Br , CN , COOH , and CHOHCH_2OH .

1 44. An implantable device according to claim 42,
2 wherein the adhesion-mediating molecule is fibronectin.

1 45. An implantable device according to claim 42,
2 wherein the support surface is titanium.

1 46. An implantable device according to claim 42,
2 wherein said proximal ends of said molecules are attached to
3 said support surface by means of binding siloxane groups.

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1 47. An implantable device according to claim 46,
2 wherein the functional group is selected from the group
3 consisting of CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOHCH_2OH , and
4 the adhesion-mediating molecule is fibronectin.

1 48. A cell growth surface comprising:
2 (a) a metallic support surface;
3 (b) a molecular monolayer of molecules having
4 proximal and distal ends, the proximal ends of said molecules
5 being attached to said support surface, said molecules of
6 said monolayer having a functional group at the distal ends
7 thereof; and
8 (c) a layer of an adhesion-mediating molecule
9 coating said monolayer, said functional group interacting
10 with the adhesion-mediating molecules of said layer to
11 provide the growth surface which tends to control the growth
12 of preselected cell types contacting said layer.

1 49. A cell growth surface according to claim 48,
2 wherein the functional group is selected from the group
3 consisting of CH_3 , $\text{CH}=\text{CH}_2$, Br, CN, COOH, and CHOHCH_2OH .

1 50. A cell growth surface according to claim 48,
2 wherein the adhesion-mediating molecule is fibronectin.

1 51. A cell growth surface according to claim 48,
2 wherein the support surface is titanium.

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1 52. A cell growth surface according to claim 48,
2 wherein said proximal ends of said molecules are attached to
3 said support surface by means of binding siloxane groups.

1 53. A cell growth surface according to claim 52,
2 wherein the functional group is selected from the group
3 consisting of CH₃, CH=CH₂, Br, CN, COOH, and CHOCH₂OH, and
4 the adhesion-mediating molecule is fibronectin.

1 54. A process for the preparation of a metallic
2 surface of an article so as to permit formation of a molecu-
3 lar monolayer on said surface, comprising contacting said
4 metallic surface with water at an elevated temperature for
5 a period of time sufficient to increase the concentration of
6 OH moieties to enable attachment of the molecular monolayer.

1 55. A process according to claim 54, wherein the
2 metallic surface is titanium.

1 56. A process according to claim 54, wherein said
2 elevated temperature is approximately 100°C.

1 57. A process according to claim 56, wherein the
2 metallic surface is titanium.

1 58. A process according to claim 54, wherein said
2 elevated temperature is at least 40°C, and further including
3 sonicating the article.

1 59. A process according to claim 58, wherein the
2 metallic surface is titanium.

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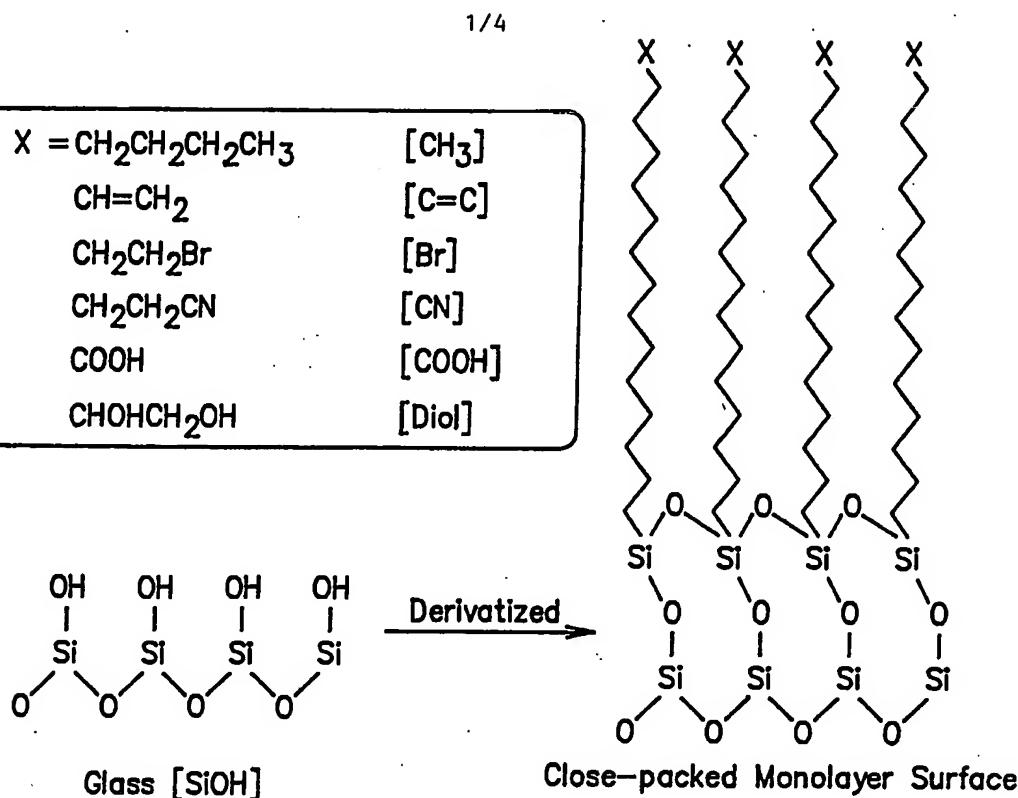


Fig. 1

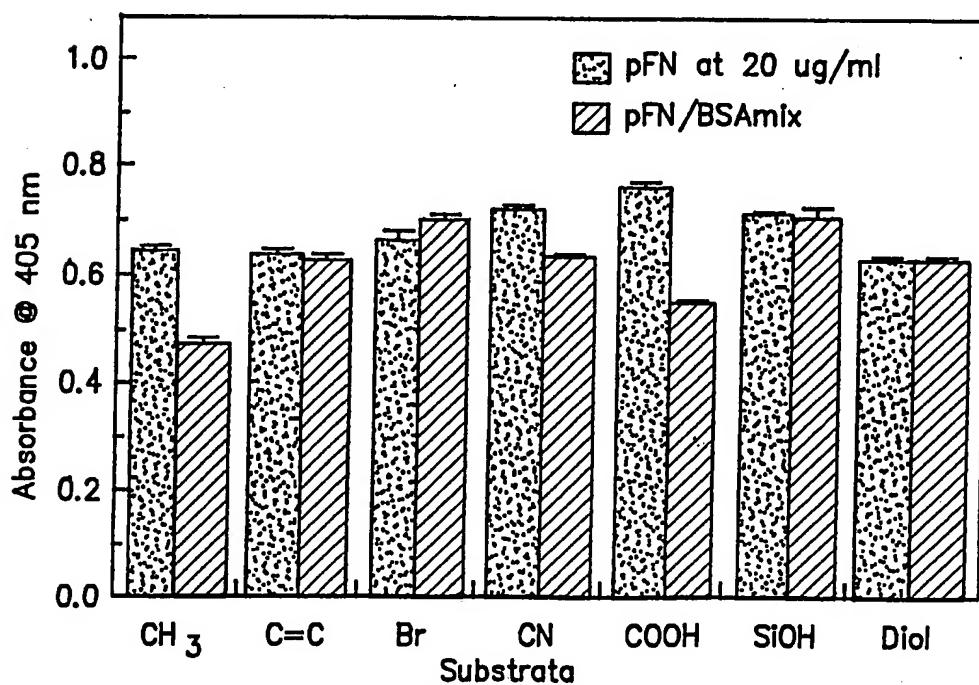


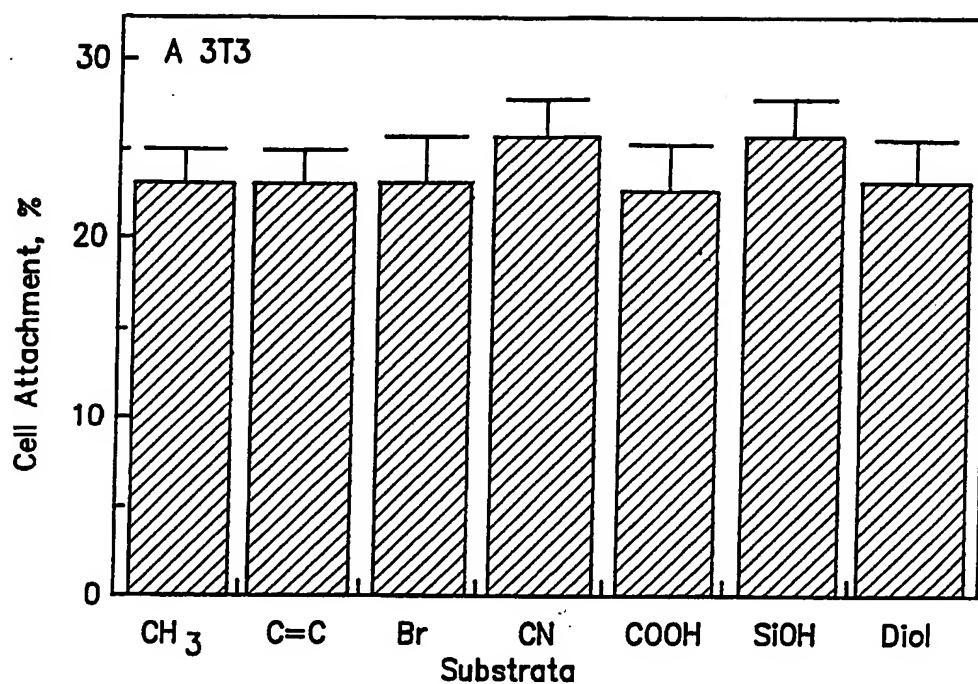
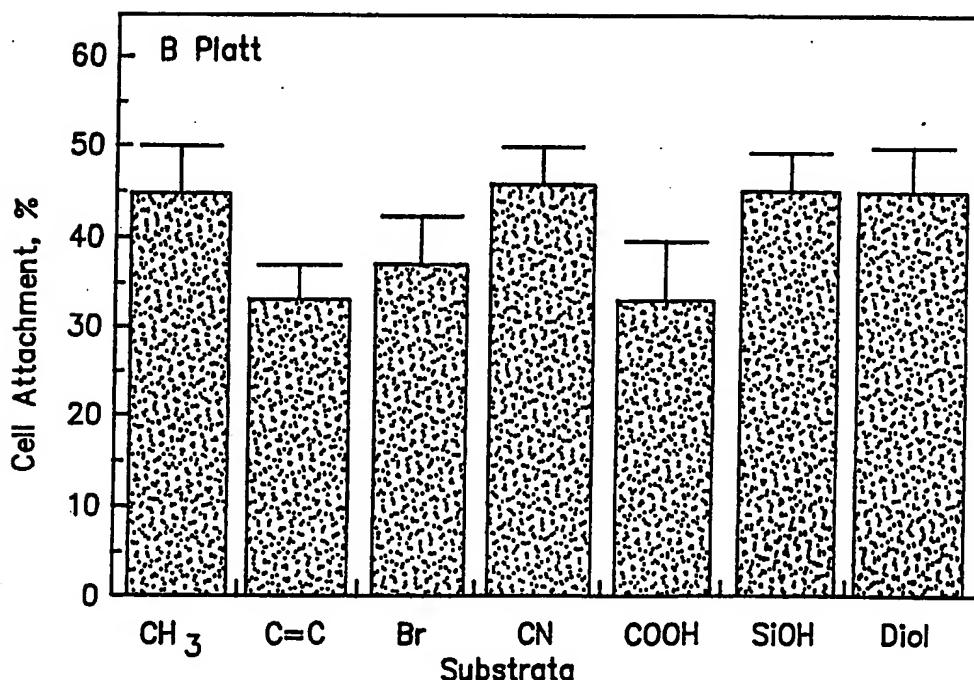
Fig. 2

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**Fig. 3A****Fig. 3B**

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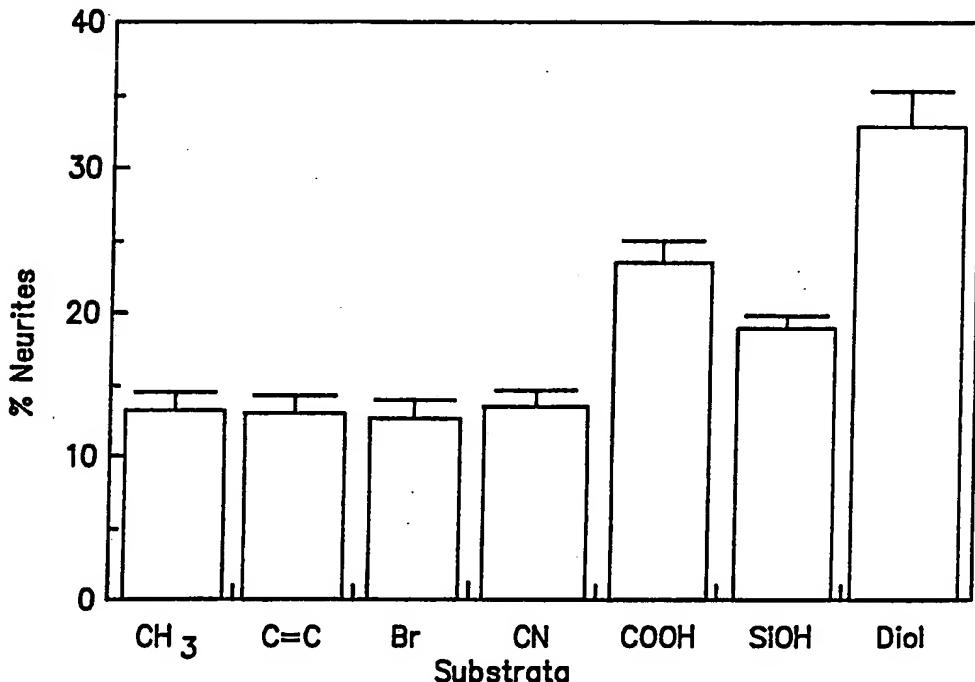


Fig. 4

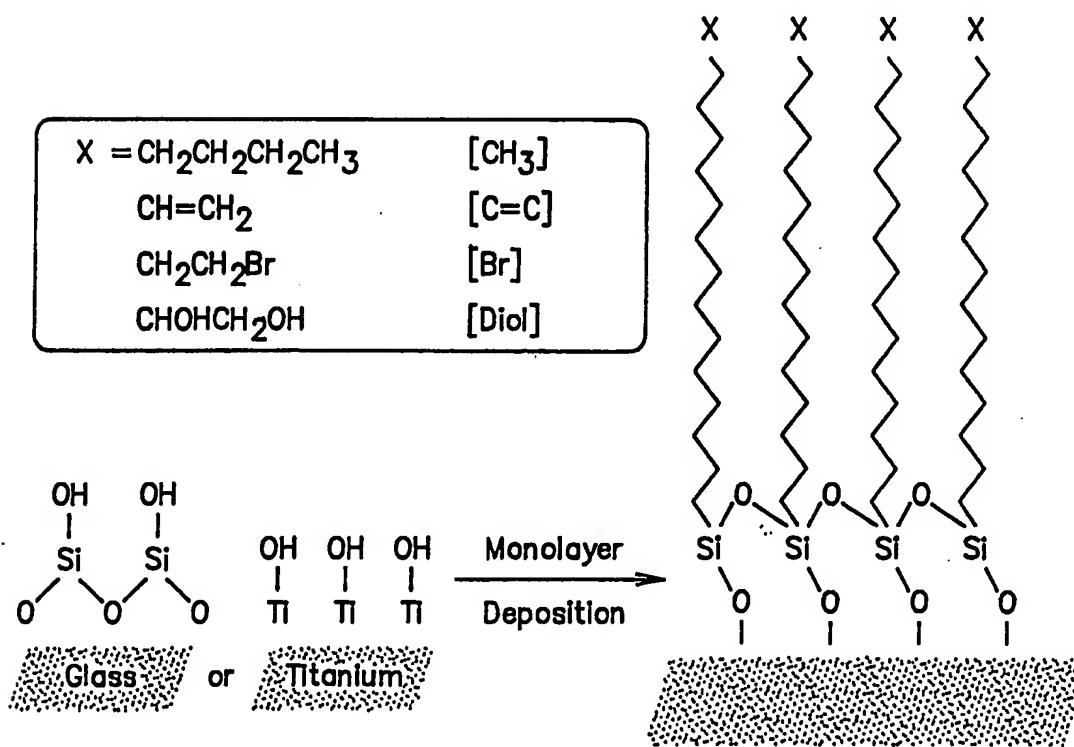


Fig. 5 Close-packed Monolayer Surface on Glass or Titanium

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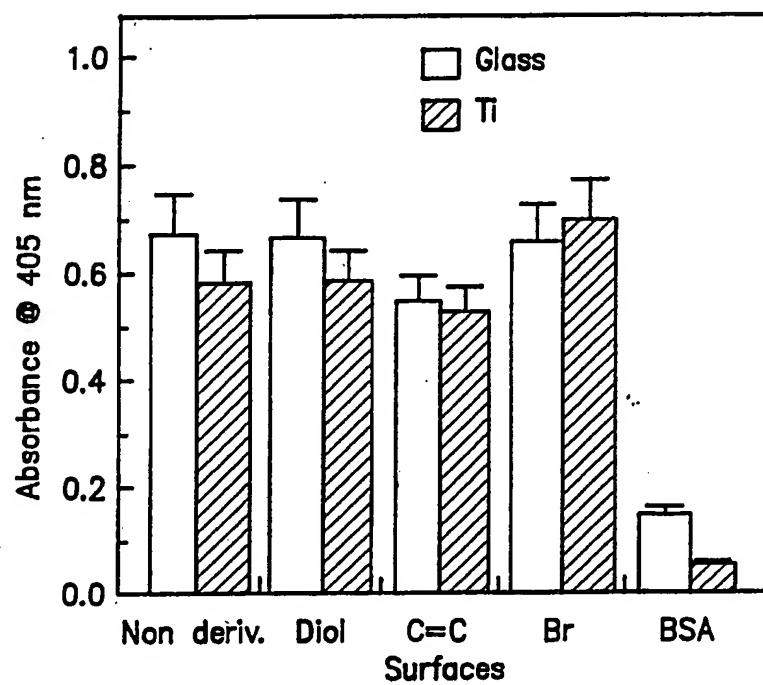


Fig. 6

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04466

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁸

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61F 2/ 54

U.S. CL.: 623/66

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S. CL.	623/11,66;427/2

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are included in the Fields Searched ⁶III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document. ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X Y	US,A, 4,878,563 (MUELLER-LIERHEIM) 09 MAY 1989; see the figure;column 1, lines 9-34;column 2, lines; 4-27; examples 1-6	1,3,4,10,14,16 17,19,30,32,33 36,38,39,42,44 45,40,50,AND 51 21-29
Y	US,A, 4,687,808 (JARRET et al.) 18 AUGUST 1987; see column 7, line 40 to column 8, line 21	21-29

* Special categories of cited documents: ¹⁰

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

16 SEPTEMBER 1991

Date of Mailing of this International Search Report

10 OCT 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

NGUYEN NGOC-HO

PAUL PREBILIC INTERNATIONAL DIVISION

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